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Diagnosis and Management of Invasive Aspergillosis in Adult Neutropenic Haemato-Oncology Patients

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Diagnosis and Management of Invasive Aspergillosis in Adult Neutropenic Haemato-Oncology Patients

Thesis submitted for the Degree of

Doctor of Medicine (Research)

King's College London

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Abstract

Background

Invasive fungal disease (IFD) is difficult to diagnose. For clinical trials the European Organisation for Research in Treatment of Cancer (EORTC) and the Mycology Study Group (MSG) criteria are useful but there are few data on their value in clinical practice. The aims of this study were to:

(1) investigate the incidence and risk factors of IFD;

(2) assess the utility of galactomannan (GM), β-D glucan (BDG), the UK consensus fungal PCR, and lateral flow device (LFD) assays together with the safety and feasibility of biopsy;

(3) assess the role of cytokines in the diagnosis and prognosis of IFD;

(4) establish the prevalence of baseline CT abnormalities, and assess diagnostic CT features and spectrum of radiological signs.

Methods

Patients (N=203) were recruited prospectively and followed for a median (range) of 556 (12-730) days after chemotherapy or haematopoietic stem cell transplantation. Chest CT, Karnofsky score (KS), serum GM, and cytokine profiles were performed at baseline; during admission twice-weekly GM assays were performed on all patients. BDG, serum and whole blood consensus PCR, and LFD assays were performed on a selection of samples from different IFD categories. Neutropenic sepsis refractory to antimicrobials for ≥4 days triggered diagnostic CT and biopsy where feasible. All patients were on

antifungal prophylaxis from admission. The revised EORTC/MSG criteria were used to diagnose IFD.

Results

The cumulative incidence of proven/probable IFD at 6, 12, and 24 months was 16, 19, and 21%, respectively. Using GM or BDG alone (plus host and clinical evidence) the apparent incidence of proven/probable IFD was 11 and 16% respectively. The median time from index treatment to onset of IFD was 142, 17, and 41 days for proven mould, proven candida and probable IFD, respectively ($P < 0.001$). The 2-year overall survival (95% CI) were 45 (29-61)% for proven/probable IFD vs. 87 (77-97)% for no evidence of IFD ($P < 0.001$). Baseline CT abnormalities were found in 76 (38%) patients, 19 of which were EORTC signs. Risk factors for IFD on multivariate Cox regression were: EORTC CT signs (Hazard ration [HR] 4.3; 95% CI 1.9-9.8; P<0.001), IL-2R >834 pg/ml (HR 2.3; 95% CI 1.1-5.1; P=0.037), MCP-1 >841 pg/ml (HR 2.7; 95% CI 1.2-6.1; P=0.016), and KS <90 (HR 2.1; 95% CI 1.1-4.2; P=0.034) at baseline as well as monocytopenia >10 days (HR 2.6; 95% CI 1.3-5.4; P=0.009) and bacteraemia (HR 2.5; 95% CI 1.2-5.0; P=0.013). The sensitivity, specificity, PPV, and NPV for GM was 54, 71, 82, and 39%, respectively; coresponding values for BDG were 79, 55, 83, and 49%. The proportion of cases identified by both tests was 38%. The sensitivity of biopsy was 35% and PCR and LFD were less sensitive but more specific than GM or BDG.

Conclusions

A multi-faceted approach is required to improve the diagnostic accuracy of IFD. No single assay was able to detect all the cases but the combination of BDG and GM seem to offer the best biomarker combination. Baseline chest

CT, KS, and cytokine profile as well as monocytopenia and bacteraemia are important risk factors. (ClinicalTrials.gov number NCT00816088).

Table of contents

List of Tables

List of Figures

Figure 67: Chemokine panel at follow-up for IFD v no IFD

Figure 68: Growth factor cytokine panel at follow-up for IFD v no IFD

List of abbreviations

AA	aplastic anaemia
ALL	acute lymphoblastic leukaemia
ALP	alkaline phosphatase
AML	acute myeloid leukaemia
AST	aspartate transaminase
BAL	broncho-alveolar lavage
BDG	β-D glucan
CLL	chronic lymphocytic leukaemia
CR	complete remission
CRP	C-reactive protein
CSA	Cyclosporin A
FA	Fanconi anaemia
FBC	full blood count
FLAMSA	fludarabine, Cytarabine, Amsacrine protocol
GGO	ground glass opacification
GM	galactomannan
GMI	galactomannan index
Hb	haemoglobin
HL	Hodgkin lymphoma
IA	invasive aspergillosis
IFD	invasive fungal disease
IPA	invasive pulmonary aspergillosis
IST	immunosuppressive therapy
ITU	intensive care unit
KOH	Potassium hydroxide

KCl	Potassium chloride
LFD	lateral flow device
LFT	liver function tests
MDS	myelodysplastic syndrome
MMF	Mycophenolate mofitil
NHL	non-Hodgkin lymphoma
OS	overall survival
PR	partial remission
SNP	single-nucleotide polymorphism
PCR	polymerase chain reaction
TLR	toll-like receptor
U&E	kidney functions tests
WBC	white blood count
VRE	vancomycin-resistant Enterococcus

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Chapter 1 Introduction and Review of Literature

1 Introduction and Review of Literature

Invasive aspergillosis (IA) is an important cause of morbidity and mortality in immunocompromised patients, particularly patients with malignant disorders undergoing high dose chemotherapy or haematopoietic stem cell transplantation (HSCT).[1-3] It is usually caused by *Aspergillus fumigatus:* a ubiquitous filamentous fungus, or mould, found in soil and decomposing vegetation which can thrive independent of animal host. *Aspergillus* species cause a wide range diseases from ABPA to chronic, saprophytic and allergic conditions but these are seldom life threatening unlike IA which is a leading cause of infection-related death in patients with acute leukaemia and those undergoing HSCT.[4] Risk factors for IA include any immunosuppression; broad-spectrum antibacterials; mucositis; quality of donor match; type of conditioning (eg total body irradiation, fludarabine); prolonged neutropenia; cytomegalovirus infection and graft-*versus*-host disease.[5] IA is encountered less frequently in solid organ transplant patients but remains a significant cause of death in this group. As improvements in chemotherapy, supportive care, antimicrobial therapy and general management have improved the prognosis for many haematological malignancies, increasingly very compromised patients are surviving longer but succumbing to IA which (in neutropenia) has a high mortality.[4,6]

However, there has been a significant decrease in mortality in patients with IA in the past two decades.[7,8] This probably reflects the changing practice in

haematology with the use of reduced intensity HSCT and increasing use of peripheral blood stem cells, both of which significantly reduces the duration of neutropenia, perhaps the main risk factor for IA. The availability of a variety of more potent antifungal agents as well as better diagnostic tools such as modern computed tomography (CT), and detection of galactomannan, β-D-glucan, and DNA by PCR allows earlier diagnosis and treatment. At the same time clinical and epidemiological studies have identified risk categories. Furthermore antifungal prophylaxis is commonly used especially in the high risk categories such as allogeneic HSCT recipients and patients undergoing induction chemotherapy for acute myeloid leukaemia (AML) or myelodysplastic syndrome (MDS).

Although these technological advances in diagnostic tools have become available to clinicians IA is still notoriously difficult to diagnose in a timely fashion as there is no universally available 'gold standard'. It is for this reason that the Invasive Fungal Infection Cooperative Group of the European Organisation for Research in Treatment of Cancer (EORTC) and the Mycology Study Group (MSG) formed a Consensus Committee in 1998 to develop standardised definitions for invasive fungal infections for clinical research and these were published in 2002 and revised in 2008.[9,10] These definitions were developed for clinical research and have not yet been validated for use in routine clinical practice. However, the availability of these diagnostic tools has allowed clinicians to move away from the fever-driven empiric antifungal therapy to a diagnostic-driven approach which could potentially save money but also reduce the unnecessary toxic treatment side effects of antifungal

therapy. However, it is unclear which of these paradigms is the superior option for the treatment of a persistently neutropenic patient.[11]

1.1 Epidemiology of IA in haematological malignancies

Aspergillus spp are ubiquitous nonpigmented, regularly septate moulds commonly found in air, soil, water, dust, compost, rotting plants, other organic debris, building construction sites.[3,12] Decomposing vegetable material is believed to be their primary ecological niche.[13] The spores are acquired primarily by inhalation where they are trapped in the upper respiratory tract and only a small proportion reaches the airways in healthy immunocompetent individuals. They cause a variety of diseases including invasive aspergillosis, allergic bronchopulmonary aspergillosis, aspergilloma and allergic pneumonitis. There are over 200 species but only a few of these are pathogenic to humans such as *A. fumigatus, A. flavus, A.niger*, and *A. terreus.* Of these, *A. fumigatus*, first described by Johann Baptist Georg Wolfgang Fresenius in 1860[14], is the commonest cause of invasive aspergillosis accounting for ~90% of IA.[2] *A. terreus*, though less common, is intrinsically resistant to amphotericin B and is associated with high mortality.[15-17]

1.1.1 Risk factors- environmental and acquired immune dysfunction

Two conditions are necessary for the development of invasive aspergillosis: simultaneous colonization and presence of risk factors.[13] In most cases the colonization occurs outside hospital settings prior to admission into hospital especially among the elderly population.[18,19] Environmental risk factors are important. Dry weather with high temperatures such as in summer and autumn are associated with higher incidence of IA.[20,21] Farmers are a particular risk

group due to inhalation of mycotoxins, endotoxins, and other toxins which colonize their airways.[22] Building reconstruction within hospitals may increase the risk of colonization and have been major causes of epidemic clusters in hospitalised patients.[12,23] The role of high efficiency particulate air (HEPA) filters to reduce air contamination is important in reducing environmental exposure.[24,25] Other possible sources of environmental contamination with spores include potted plants, flowers, carpets, and water distribution systems (as spores get released during showering).[26,27]

The risk factors related to impaired host defences may be acquired in the majority of cases or genetic in some (Table 1). Prolonged and profound neutropenia due to any cause is the most important risk factor for IA.[28-33] This is not surprising given that neutrophils are the main effector cells involved in the phagocytosis and killing of *Aspergillus* spores. Dysplastic neutrophils in MDS are less fungicidal than normal neutrophils.[34] Lymphocytopenia and T-cell dysfunction is also a risk factor following allogeneic HSCT.[35,36] Interestingly adoptive transfer of anti-*aspergillus* T- cells may be effective in treating IA in the haploindentical HSCT setting.[37]

In allogeneic HSCT the quality of donor-recipient HLA match, the source of stem cells, type of conditioning regimen used, T-cell depletion, and CMV reactivation, and presence of acute or chronic graft versus host disease (GVHD) are associated with increased risk of IA.[30,38] Recipients of cord blood transplantation are a particularly high risk group probably reflecting the naivety

of the immune cells in cord blood and also the slower immune recovery post-transplant.[39,40] In recipients of allogeneic stem cell transplantation a bimodal risk period is recognised. The early phase is characterised by neutropenia and damaged mucosal barrier which may be aggravated by acute GVHD during the first 100 days.[41] The late phase is characterised by chronic GVHD, CMV reactivation, and treatment with steroids and other immunosuppressive agents.[35,38,42-45] T-cell depletion reduces the incidence of GVHD but impairs reconstitution of cellular immunity and CMV reactivation.[44]

Table 1: Risk factors for IA in haemato-oncology patients (modified from[8,46-49])

Acquired	**Genetic**
Prolonged neutropenia	TLR polymorphisms
HSCT Older age at transplant Underlying disease Type of transplant T-cell depleted or CD34-selected Cord blood transplant Unrelated or HLA-mismatched PBMC CMV reactivation GVHD	Plasminogen SNP Mannose-binding lectin (MBL) IL-1 SNP IL-10 promoter gene polymorphism TNF-R2 promoter VNTR
Immunosuppressive therapy: cyclosporine CSA), tacrolimus, mycophenolate mofitil (MMF), antithymocyte globulin (ATG)	
Corticosteroids	
Lymphocytopenia, monocytopenia T-cell dysfunction eg Alemtuzumab therapy, anti-TNF therapies, anti-CD3 antibodies	
Diabetes	
AIDS	
Iron overload	

The dose and duration of corticosteroids used in the treatment of GVHD is important.[38,46,50] Steroids have multitude of effects on the immune system

such as prevention of killing of phagocytosed *A. fumigatus*[51], blunting the alveolar macrophage production of proinflammatory cytokines such as IL-1a, TNF-α, and macrophage inhibitory factor 1α[52], and Th-2 polarization[53,54]. The new anti-neoplastic or immunosuppressive agents especially purine analogues (eg fludarabine) and antibodies targeted against immune cells such as anti CD52 (alemtuzumab), anti-thymocyte globulins (ATG), and anti-CD3 antibodies, may produce transient neutropenia as well as prolonged lymphocytopenia and impairment of cell mediated immunity[55,56]. Similarly anti-TNF therapy (eg infliximab and etanercept) for treatment of refractory GVHD or for non-haematological indications such as rheumatoid arthritis is associated with increased incidence of IA[57]. IA acquired in the pre-transplant chemotherapy phase is an important risk factor as the risk of relapse during transplant in such patients is extremely high approaching 30%.[45]

Iron overload, as assessed by marrow iron stores, has been recently identified as a risk factor for IA[58] Transfusion-dependent MDS and AML patients are particularly at risk. Iron is an important element for a variety of biological pathways and in *Aspergillus* spp iron acquisition is essential for increased growth and virulence[59] Interestingly the iron chelator deferoxamine is associated with increased iron uptake by certain moulds such as zygomycetes which use it as a siderophore to access previously unavailable iron and this uptake is associated with fungal growth.[60] The newer iron chelators deferiprone and deferasirox do not act as siderophores for the fungus.[61]

1.1.2 Genetic risk factors

The role of innate immune receptors such as the toll-like receptor (TLR) family in regulating both innate and adaptive immune responses to *Aspergillus* infection has attracted attention. Bochud et al have shown that single nucleotide polymorphisms (SNPs) in TLR2 and TLR4 (S4 haplotype) of HSCT donors rendered recipients more susceptible to IA by more than two fold.[62] The presence of TLR1 and TLR5 polymorphisms in recipients has also been shown to increase the risk of IA post- transplant.[63] Other genetic risk factors include SNPs in the plasminogen gene[64] and possibly mannose binding lectin (MBL) gene.[65] Recent studies have also identified dectin-1 mutation as a novel risk factor for IA.[66]

Cytokine gene polymorphisms are also associated with increased risk of IA. Interleukin 10 (IL-10) is an important regulatory cytokine that inhibits the inflammatory effector functions of macrophages and dendritic cells. Seo et al[67] investigated 105 patients undergoing HSCT and examined three SNPs in the proximal region of the IL-10 promoter. They found that ACC haplotype, which is associated with decreased IL-10 production, was associated with a 9-fold decrease in the risk of developing IA. On the other hand increased IL-10 production, as in the ATA haplotype, was associated with a higher incidence of IA. Other cytokine gene polymorphisms associated with increased risk of IA include variable number of tandem repeats (VNTRs) at position -322 of the TNFR2 gene[68] and IL1 gene cluster polymorphisms.[69]

1.1.3 Incidence/prevalence of IA

Taking the above risk factors into account it is possible to stratify patients into low, intermediate and high risk categories for IA (Table 2)

Table 2: Risk stratification of patients with haematological malignancies for IA (modified from [49])

Low risk	Intermediate risk	High risk
Autologous HSCT	Acute Lymphoblastic Leukaemia	AML (particularly during induction chemotherapy)
Hodgkin lymphoma	Chronic Lymphocytic Leukaemia	Allogeneic HSCT (especially cord blood)
Chronic Myeloid Leukaemia	Myelodysplastic Syndrome	
Chronic myeloproliferative disorders	Non Hodgkin lymphoma	
Multiple myeloma		

After World War II there has been a steady rise in the incidence of invasive fungal infections in general and invasive aspergillosis in particular, primarily as a consequence of the use of more intensive chemotherapy for treatment, use of HSCT, and the better supportive care which makes it possible for increasingly immunosuppressive patients to survive longer than before.[70] At the same time the widespread use of mould-active prophylactic antifungal agents has changed the epidemiology of invasive mould infections so that non-*fumigatus Aspergillus* species such as *zygomycetes*, and *fusarium* have increased in relative terms.[71,72]

Pagano et al performed a multicentre retrospective cohort study in Italy (SEIFEM-2004 study) between 1999 and 2003 on 11,802 patients with haematological malignancies and they found IA incidence of 2.6%.[73] The highest incidence was found in patients with AML (7.9%) particularly during the first induction chemotherapy when 43% of these cases were diagnosed. The diagnosis of IA was probable in 60% and proven in 40% using the 2002 EORTC/MSG criteria [9] and *A. fumigatus* was identified in 53% of proven cases. It is noteworthy that this study excluded patients with MDS, aplastic anaemia and those undergoing HSCT. The incidence of IA in patients with acute leukaemia and MDS is variable in different studies but it is around 5-10%.[74,75] This risk increases with multiple cycles of chemotherapy. The second Italian study (SEIFEM B-2004) focussed on HSCT patients. Amongst 3228 patients 1249 were allogeneic and 1979 were autologous transplants.[76] The incidence of IFI was 7.8% and 1.2% among allogeneic and autologous recipients respectively.

A large retrospective US autopsy study was performed on 1017 patients who had chemotherapy or HSCT over a 15 year period between 1989 and 2003 in the M.D. Anderson Cancer Center by Chamilos et al.[77] They found invasive fungal disease (IFD) in 31% of patients and 17% had IA. The majority (75%) of these cases were diagnosed post mortem. Patients with acute leukaemia and those undergoing HSCT had the highest prevalence of IA.

The incidence of IA in autologous HSCT is low and is estimated to be 0.3-2%.[45,76,78-80] Allogeneic HSCT is a higher risk procedure and the overall incidence is estimated to be 2.9-15%.[31,45,50,80-85] The second at risk phase of IA post allograft poses a major challenge with cumulative incidences of up to 15% at 12 months post- transplant.[86] Pre-transplant, IA is another challenge due to the high of reactivation during the early or late phase post-transplant. The recurrence risk is associated with short duration (<1 month) of antifungal therapy before transplantation, persistence of radiological abnormalities and myeloablative conditioning, especially, total body irradiation-containing regimens.[45] Martino et al evaluated 129 cases of proven or probable IA diagnosed prior to transplantation.[87] They observed progression of IA in 27 (17%) with cumulative incidence of 22% at 2 years post-transplant. This cumulative incidence was related to longer duration of neutropenia post allograft, advanced status of underlying disease, short duration of antifungal therapy (less than 6 weeks), myeloablative conditioning, CMV disease, donor source (bone marrow or cord blood), and presence of acute severe GVHD.

Recently a network of 23 US transplant centres, the Transplant Associated Infections Surveillance Network (TRANSNET), prospectively enrolled 875 HSCT recipients with proven and probable IFIs occurring between March 2001 and March 2006.[88] The median onset of IA was 99 days post HSCT and the 12-month cumulative incidence of IA was 1.2% for autologous HSCT and for allogeneic HSCT it was 5.8%, 7.7%, and 8.1% for matched related donors, matched unrelated donors, and mismatched matched donors respectively.

However, there was a wide variation between the sites with incidences ranging from 3.1 to 20.6% for mismatched related donors.

1.1.2 Prognostic factors

The immune system and the underlying malignancy and its treatment are the primary factors that determine the outcome of patients with IA.[49] In general the prognosis for patients with haematological malignancy who develop IA is poor. Lin et [7] carried out a large systematic review of literature and found that the overall case fatality rate was 58% in patients with malignancy but in those who had allogeneic HSCT or central nervous system involvement or disseminated IA it was approximately 90%. This has been confirmed by more recent studies showing mortality of 77-90% after allogeneic HSCT [8,76], 42% after chemotherapy [73], and 17% after autologous HSCT.[76] The main adverse prognostic factors are summarised below [49]:

1. Neutropenia

Prolonged neutropenia post allograft or chemotherapy is associated with poor outcome.[46,89-91] This is usually due to progression or refractoriness of underlying disease.

2. Progression of underlying disease [91]
3. Monocytopenia
4. Disseminated disease especially with CNS involvement
5. Renal and hepatic insufficiency
6. GVHD and its treatment especially with high doses of steroids
7. Persistently high serum galactomannan (GM) levels

In a recent study Koo et al evaluated 93 patients with proven and probable IA and showed that persistently high GM during antifungal therapy is associated with poor outcome.[92] They concluded that GM at diagnosis and GM decay in the week following diagnosis are independent risk factors for mortality. In addition, the management strategy adopted may influence outcome. Treatment with voriconazole or lipid formulations of amphotericin B is associated with improved prognosis.[8,46,90,91,93] It is also important to initiate effective antifungal therapy early in patients with IA as delay may worsen prognosis.[49]

1.2 Pathogenesis: infection and the host immune response

Asexual reproduction is the primary form of reproduction by *Aspergillus* species and proceeds on specialised structures called conidiophores where the asexual spores (conidia) that easily form aerosols are produced.[94] When inhaled, small conidia (2.0-3.5 µm in diameter), such as those of *A. fumigatus* may traverse the whole length of the respiratory airways down to the alveolar spaces whereas larger conidia of *A. flavus* and *A.niger* settle in the upper airways and paranasal sinuses. Effector cells of the innate immune system responds to eliminate the conidia: alveolar macrophages phagocytose and intracellularly destroy conidia by releasing NADPH oxidase-dependent reactive oxidant intermediates (ROI), neutrophils aggregate around the conidia and prevent their germination by releasing lactoferrin from their primary granules, and platelets release serotonin from their dense granules.[51,95,96] The respiratory mucosa augments the immune response by secretion of antimicrobial peptides, such as surfactant proteins, lactoferrin, lysozyme and defensins.[97,98] In effect the innate immune response to fungi has two objectives:

1. pathogen destruction by phagocytosis or secretion of antimicrobial agents against undigestible fungal elements and

2. instructive role to the adaptive immune system through pro-inflammatory cytokines and chemokines, the induction of co-stimulatory activity by phagocytic cells and antigen uptake and presentation.[36]

The role of macrophages as the first line of defence was first described in a guinea pig model by Gernez-Rieux and colleagues.[99] and later confirmed by Schaffner et al.[100] Schaffner et al [100] gave an excellent description of macrophages as the first line of defence against spores and neutrophils as the second line of defence against hyphae and concluded:

'*The host, thus, can call upon two independent phagocytic cell lines that form graded defence systems against aspergillus. These lines of defence function in the absence of a specific immune response, which seems superfluous in the control and elimination of this fungus'*.

In addition Schaffner et al identified the role of corticosteroids in invasive aspergillosis due to their inhibition of the conidiacidal activity of macrophages.

In the immunocompromised patients the inhaled conidia germinate, a process that involves conidial swelling, to produce hyphae capable of tissue invasion. This is due to the fact that unlike conidia, hyphae are too large for macrophage engulfment and are targeted principally by Neutrophils.[101] Activated neutrophils aggregate around hyphae and extracellularly secrete reactive oxygen species (ROI) and other antimicrobial peptides.[102,103] Dying neutrophils also form neutrophil extracellular traps (NETs), composed of chromatin and DNA covered with granular fungicidal proteins.[104]

The innate immune system relies on a limited repertoire of germ line-encoded receptors called pattern recognition receptors (PRR) on the surface of immune cells or in biological fluids to recognise pathogen-associated molecular patterns (PAMP), common molecular motifs shared by a large group of

microorganisms.[105] The best characterised class of cellular PRR involved in the recognition of *Aspergillus* are Toll-like receptors (TLR) and lectin receptors (such as dectin-1). Soluble PRR include collectins (mannose-binding lectin [MBL], surfactant proteins, C1q) pentraxin (PTX) and ficolin.[36] The primary structure of the *Aspergillus* cell wall is composed of a complex meshwork of β-glucan, galactomannan and chitin presenting potential targets for PRR binding.[106] These result in the induction and propagation of inflammatory response to *Aspergillus* and indeed other fungi. For instance, binding of the soluble receptor long pentraxin-3 (PTX3) to conidial galactomannan mediates, through opsonization, conidial uptake by alveolar macrophages and dendritic cells, resulting in the production of protective T helper cell type 1 (Th1) cytokines.[107] PTX3-null mice have increased susceptibility to IPA [107] and the administration of PTX3 protects against IA in a murine model of allogeneic bone marrow transplantation.[108]

1.2.1 TLR-PAMP system

TLRs are membrane signalling receptors that collectively recognise lipid, carbohydrate, peptide and nucleic-acid structures which are broadly expressed by a variety of microorganisms. However, *Aspergillus*-specific ligands for these PPRs are unknown. All TLRs, on binding PAMPs, stimulate a core of stereotypic responses but individual TLRs can induce specific responses which are either myeloid differentiation primary response gene 88 (MyD88)-dependent [109] or independent.[110] A major downstream effect of TLR signalling is the activation of nuclear factor-κB (NF-κB) which is an important transcription factor required for the expression of many genes related to innate

immunity and inflammation [111] (Figure 1). TLR2 and TLR4 mediate *Aspergillus fumigatus* recognition by macrophages.[112,113]

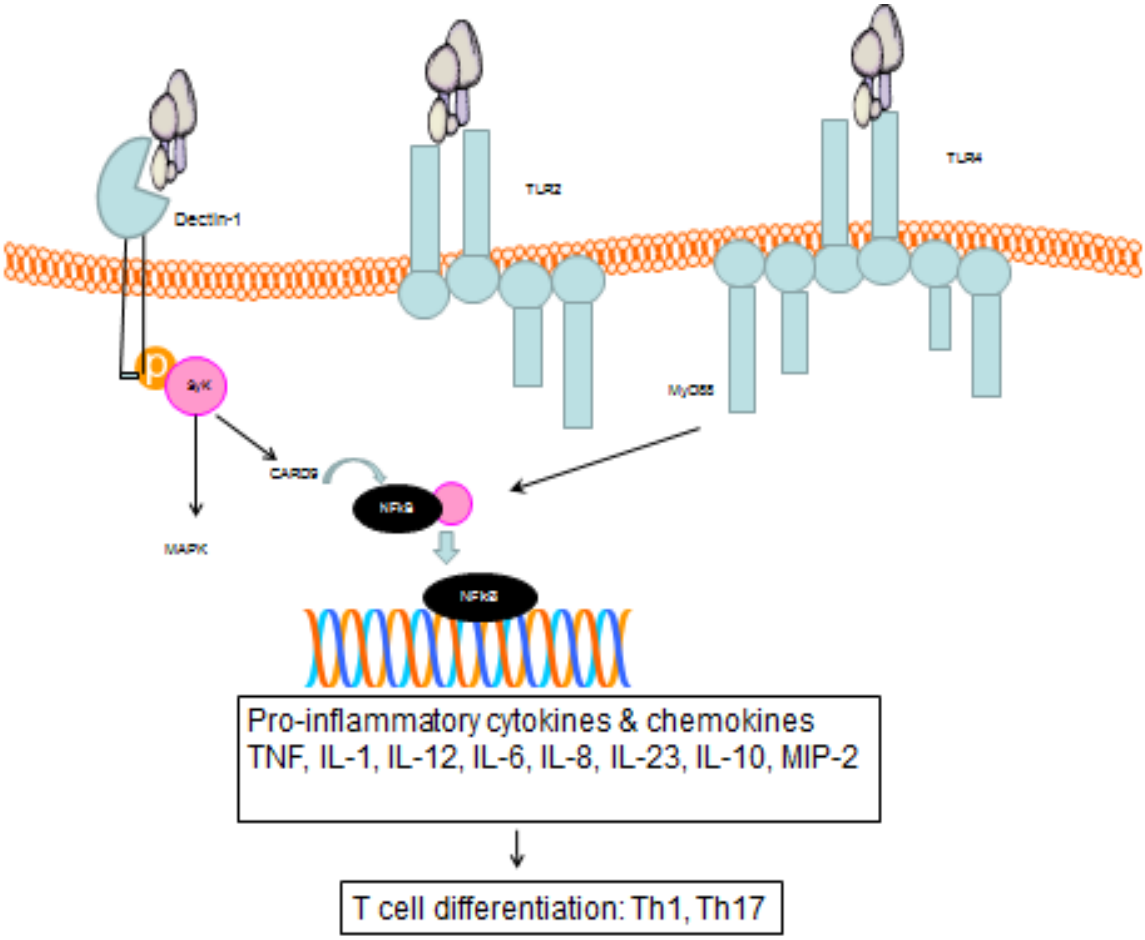


Figure 1: Model for immune response to fungal infection. Fungi such as *Aspergillus fumigatus* (represented by ) are recognised by dendritic cells and other immune cells via their receptors such as TLR and Dectin-1 which triggers a cascade of intracellular reactions leading to activation of NF-κB, an important transcription factor that leads to transcription of key pro-inflammatory cytokine genes (adapted from Rivera & Pamer [114]).

1.2.2 β-Glucan recognition

β-glucans are naturally occurring polysaccharides that form a major constituent of the fungal cell wall and play an important role in antifungal immune responses. They are recognised by a PRR called dectin-1, which belongs to a family of calcium-dependent carbohydrate binding proteins expressed on the plasma membranes of macrophages, dendritic cells and other white blood cells.[115-117] Dectin-1-β-glucan-binding starts a cascade of intracellular signalling leading to activation of NFκB. This in turn produces cytokines that help drive CD4+ T-lymphocytes towards a Th1 and Th-17 phenotype.[36,118,119] The latter is a signal transducer and activator of the transcription (STAT-3) dependent process.[120] Th17 lymphocytes produce IL-17 and IL-22 which augments neutrophil production and recruitment, and production of antimicrobial proteins by epithelial cells respectively.[120] Mutations of genes affecting this cascade have been shown to cause various fungal susceptibility syndromes such as Job's (Hyper-IgE) Syndrome (STAT3 mutation), and recurrent mucocutaneous candidiasis (Dectin-1 and CARD-9).[121,122] More recently the Dectin-1 polymorphisms (Y238X, rs6910526), which causes a change in amino acid 238 from tyrosine to an early stop codon leading to a truncated carbohydrate recognition domain (CRD) of dectin-1, has been shown to be an important risk factor for IA.[66] Cunha and colleagues studied 205 adult patients with haematological malignancies who underwent T-deplete allogeneic HSCT and found that the presence of Dectin-1 Y238X polymorphism in either donors or recipients increased susceptibility to IA with the highest risk present in cases of simultaneous polymorphism in both donors and recipients.[66]

Dectin-1 activates the transcription of NFκB through the canonical and non-canonical pathways, the only known PRR to do so.[123] The NFkB family are key transcription factors and consist of five members which can homo- or hetero-dimerise: p65, RelB, c-Rel, p50, and p52.[124] The canonical pathway involves the phosphorylation of spleen tyrosine kinase (Syk) which induces an assembly of a protein scaffold consisting of caspase recruitment domain 9 (CARD9), Bcl-10 and MALT1 which activates IkB Kinase (IKK) complex, leading to nuclear translocation of NFkB subunit p65 and c-Rel.[125] The non-canonical pathway is also Syk-dependent and results in the activation of NFκB subunit RelB and p52 which down regulates IL-12 transcription.[123,124] Dectin-1 also activates a Syk-independent pathway through the serine-threonine kinase, Raf-1 which represses the Syk-induced RelB activity but increases p65 transcriptional activity to induce the Th1 and Th-17 phenotypes.[118,123]

The innate immune system has the ability to distinguish between the resting and germinating conidia by a stage-specific β-glucan display.[113,126] In the dormant conidia, β-glucan is shielded by an immunologically inert hydrophobic RodA protein covalently bound to the fungal cell wall.[127] This immunological silencing of the resting fungal spores is consistent with previous observations that dectin-1 does not recognise resting conidia but instead recognises swollen and germinating conidia and is important in preventing excessive and potentially detrimental inflammatory host responses. TLR response to *Aspergillus* is also stage dependent: TLR2 is activated by both conidia and hyphae while TLR4 is only activated by conidia.[128] This may be an important escape mechanism for germinating *Aspergilli*. Other immunomodulatory

mechanisms include the suppression of TLR2- and TLR4-induced IL-1β and IL-6 production by TLR2 internalisation with the conidia into phagosomes which results in a decrease in the surface receptor expression whereas hyphae selectively downregulate TLR4-mediated responses.[129] It is suggested that TLR2 activation in the absence of TLR4 may skew host response to a Th-2 type cytokine response which may impair effective host resistance to fungal infection.[130,131]

1.2.3 Cytokine production in IA

Cytokines are polypeptides secreted by cells of the innate and adaptive immune systems in response to fungal antigens that mediate and regulate effector functions of the immune cells. Typically their actions are brief and self-limited with inhibitory feedback mechanisms to turn down their responses and their actions may be local or systemic. They initiate their actions by binding to receptors on target cells. They can be divided into 3 main functional categories[132]:

1. mediators and regulators of innate immunity produced by mononuclear phagocytes in response to infectious agents that stimulate their PRR (Table 3 & 4)

2. mediators and regulators of adaptive immunity (Table 5)

3. growth factors in particular haematopoiesis (Table 6)

Chemokines (‘chemotactic cytokines’) stimulate and regulate leukocytes movement from blood to tissues. They consist of 8-12 kD poplypeptides containing two internal disulphide loops and are classified into 4 groups based

on the number and location of N-terminal cysteine residues.[132] The CC (cysteine residues are adjacent) and CXC (cysteine residues are separated by one amino acid) families are the commonest and most important.

Activation of PRRs induces the maturation of antigen presenting cells which facilitate the development of adaptive T helper (Th) cell-based immune response through the production of specific set of cytokines.[36] These helper T cells have been traditionally divided into Th1 and Th2.[133] Th1 cells are essential for defence against intracellular pathogens such as bacteria and fungi and produce large quantities of interferon-γ as their signature cytokine which activate macrophages and induce delayed hypersensitivity reactions (Table 7). Th2 cells function primarily to clear parasitic infections by mainly producing IL-4, inducing IgE production and recruiting eosinophils and mast cells to the site of infection. More recently two further lineages have been described: Th17 and regulatory T cells (Treg).[134,135] In the immunocompetent host the Th1 cytokine profile as well as the Th17 cytokines (IL-17A, IL-17F, IL-22) provide robust antifungal responses at cellular as well as mucosal surfaces.[120,136] An increase in Th1 cytokines versus Th2 cytokines has been shown to lead to improved outcomes in patients with IA.[54] In mice rapid Th1 differentiation of *A. fumigatus*-specific CD4+ cells occur during IPA in the lymph nodes draining the lungs and that these cells become fully differentiated IFN-γ producing CD4+ T cells upon arrival in the lungs.[137] This process is TLR and MyD88-dependent in the lymph nodes but not in the lungs.[137]

Table 3: Cytokines of the innate immune system (adapted from [132])

Cytokine	Main cell source	Stimulus	Biological functions
Tumour necrosis factor (TNF)	**Macrophages** Activated T cells, NK cells, mast cells	TLR engagement INF-γ	• Neutrophils/monocytes→activation • Endothelial cells→ activation (inflammation & coagulation • Hypothalamus: fever • Liver: synthesis of acute phase proteins • Muscles/fat: catabolism • Other cells: apoptosis
Interleukin-1 (IL-1)	**Macrophages**, neutrophils, endothelial cells, epithelial cells	TLR engagement TNF	• Endothelial cells→ activation (inflammation & coagulation • Hypothalamus: fever • Liver: synthesis of acute phase proteins
Interleukin-12 (IL-12)	Macrophages DCs	TLR signalling INF-γ	• T cells:Th1 differentiation • NK cells & T cells: IFN-γ synthesis, increased cytotoxicity
Type 1 interferons (INF-α, INF-β)	INF-α: plasmacytoid dendritic cells, macrophages INF-β:fibroblasts	Viral nucleic acids	• All cells: increased MHC class 1 expression and inhibit viral replication • NK cells: activation • T cells:Th1 differentiation
Interleukin-10 (IL-10)	Macrophages, Treg cells, neutrophils, DCs	Macrophage activation	• Macrophages & DCs: inhibition of effector functions (IL-12, TNF, IL-6, etc) and expression of costimulators and class II MHC molecules
Interleukin-6 (IL-6)	Macrophages, endothelial cells, T cells, activated T cells	Microbes, IL-1, TNF	• Liver: acute phase proteins • Bone marrow: synthesis of neutrophils
Interleukin-15 (IL-15)	Macrophages, others	Viral nucleic acids, LPS,	• NK cells: proliferation (and perhaps differentiation & activation) • T cells: proliferation, survival of memory CD8+ T cells
Interleukin-18 (IL-18)	Macrophages, DCs	Intracellular microbes	• NK cells & T cells: IFN-γ synthesis, Th1 differentiation (synergy with IL-12)
Chemokines	See Table 2		

NK, natural killer; DC, dendritic cell; MHC, major histocompatibility complex; TLR, toll-like receptor; LPS, lipopolysccharide

Table 4: Chemokines (adapted from [132])

Chemokine	Original name	Chemokine receptor	Functions
CC chemokines			
CCL2	MCP-1	CCR2	mixed leukocyte recruitment
CCL3	MIP-1α	CCR1, CCR5	mixed leukocyte recruitment
CCL4	MIP-1β	CCR5	T cell, DC, monocyte and NK recruitment HIV coreceptor
CCL5	RANTES	CCR1, CCR3, CCR5	mixed leukocyte recruitment
CCL11	Eotaxin	CCR3	Eosinophil, basophil and Th2 recruitment
CXC chemokines			
CXCL8	IL-8	CXCR1, CXCR2	Neutrophil recruitment
CXCL9	Mig	CXCR3	Effector T cell recruitment
CXCL10	IP-10	CXCR3, CXCR3B	Effector T cell recruitment
CXCL12	SDF-1α/β	CXCR4	Mixed leukocyte recruitment HIV coreceptor

Table 5: Cytokines of the adaptive immune system (adapted from [132])

Cytokine	Main cell source	Stimulus	Functions
Interleukin-2 (IL-2) (Previously called T cell growth factor)	T cells (mainly CD4+ T cells)	Intracellular microbes	T cells: proliferation & survival (induces anti apoptotic protein Bcl-2), ↑cytokine synthesis; Treg development & survival NK cells: proliferation, ↑cytotoxicity B cells: proliferation, antibody synthesis (*in vitro*)
Interleukin-4 (IL-4)	CD4+ T cells (Th2) Mast cells	Helminthic parasites	B cells: isotype switching to IgE & IgG4 T cells: Th2 differentiation, proliferation, inhibition of Th1 & Th17 differentiation Macrophage: inhibition of IFN-γ-mediated activation
Interleukin-5 (IL-5)	CD4+ T cells (Th2)	Helminthic parasites	Eosinophils: activation, ↑production B cells: proliferation, IgA production (*in vitro*)
Interleukin-13 (IL-13)	CD4+ T cells (Th2), NKT cells, mast cells	Helminthic parasites	B cells: IgE class switching Epithelial cells: ↑mucus production, inflammation Fibroblasts: ↑collagen synthesis Macrophages: ↑collagen synthesis (through TGF-β production)
Interferon-γ (INF-γ)	T cells (Th1, CD8+ T cells) NK cells	Antigen recognition, IL-12 and IL-18	Macrophages: activation, ↑microbicidal activity B cells: isotype switching to opsonising and complement-fixing IgG T cells: Th1 differentiation (via T-bet) Others: ↑expression of class I & II MHC molecules, ↑antigen processing and presentation to T cells
Transforming growth factor-β (TGF-β)	Many cells	Antigen stimulated T cells	T cells: ↓proliferation and effector functions; Treg differentiation B cells: ↓proliferation; IgA production Macrophages: ↓activation; stimulation of angiogenic factors Fibroblasts: ↑collagen synthesis
Interleukin-17 (IL-17)	T cells	TGF-β + IL-6 (+IL21 & IL-1β)	Endothelial cells: ↑chemokine production Macrophages: ↑chemokines & cytokine production Epithelial cells: GMCSF & GCSF production
Interleukin-6 (IL-6)	Macrophages, endothelial cells, T cells	Microbes, IL-1, TNF	B cells: plasma cell differentiation T cells: Th17 differentiation, inhibition of Treg

Table 6: Cytokines of haematopoiesis and other growth factors (adapted from [132])

Cytokine	Main cell sources	Principal cell target/cell population induced
Stem cell factor (c-Kit ligand)	BM stromal cells	Pluripotent stem cells: all
Interleukin-7 (IL-7)	Fibroblasts, BM stromal cells	Immature lymphoid cells: B & T lymphocytes
Interleukin-3 (IL-3)	T cells	Immature progenitors: all
Granulocyte-monocyte CSF (GM-CSF)	T cells, macrophages, endothelial cells, fibroblasts	Immature and committed progenitors, mature macrophages: granulocytes, macrophage activity
Granulocyte CSF (G-CSF)	Macrophages, fibroblasts, endothelial cells	Committed progenitors: granulocytes
Interleukin-6 (IL-6)	Macrophages, endothelial cells, T cells	Immature progenitors: all
Epidermal growth factor (EGF)		Epidermal and epithelial cells
Vascular endothelial growth factor (VEGF)	Vascular endothelial cells	Vascular endothelial cells→ angiogenesis
Fibroblast growth factor-basic (FGF-b)	Endothelial cells	Mesenchymal cells, endothelial cells→ vasculogenesis, angiogenesis, haematopoieisis, wound healing
Hepatocyte Growth factor (HGF)	Mesenchymal cells	Epithelial cells, endothelial cells, Immature and committed progenitors→ growth and motility

The role of Th17 in controlling IA is becoming clearer. IL-17 is important in the recruitment of neurophils, whereas, IL-22 induces epithelial production of the cationic antimicrobial peptides β-defensin 2 and 3.[136] The controversy surrounding the role of Th17 is due to the fact that it encourages inflammatory tissue damage and on its own promotes, rather than controls, IA.[138] In fact successful clearance of IA is dependent on robust Th1 response coupled with a weak Th17 response.[94] This may be due to the fact that *A. fumigatus* possesses indoleamine 2,3-deoxygenase (IDO) which metabolises tryptophan into kynurenine, an immunoregulatory catabolite which inhibits Th17 and induces regulatory T cells.[139] Polymorphisms of some cytokine genes have also been described which provide an additional layer of susceptibility to IA such as CXCL-10 [140], IL-10 promoter [67,141], IL-1 [69] and variable number of tandem repeats of TNF receptor type 2 promoter.[68] These polymorphisms together with the Dectin-1 and TLR polymorphisms described above may become an important tool for risk stratification of patients undergoing HSCT or high dose chemotherapy.

Table 7: Properties of the Th1 and Th2 CD4 T helper cells (adapted from [132])

	Th1 profile	**Th2 profile**
Cytokines produced		
IFN-γ	+++	-
IL-4, IL-5, IL-13	-	+++
IL-10	+/-	++
IL-3, GM-CSF	++	++
Cytokine receptor expression		
IL-12R β chain	++	-
IL-18R	++	-
Chemokine receptor expression		
CCR4, CCR8, CXCR4	+/-	++
CXCR3, CCR5	++	+/-
Immune system promoted	Cell mediated	Humoral
Main partner cell	Macrophage	B cell

1.2.4 Angioinvasion

The majority of inhaled *A.fumigatus* conidia that reach the pulmonary alveoli are destroyed by type II pneumocytes, but about 3% of them survive within pneumocytes[142]. The process of angioinvasive aspergillosis is thought to occur via the following steps [143]:

Within pulmonary alveoli

1. Inhalation of airborne conidia
2. Adherence of conidia to pulmonary epithelial cells

3. Endocytosis of conidia by epithelial cells primarily type II pneumocytes. Endocytosed live but not killed *A. fumigatus* conidia actively inhibit apoptosis, induced by TNF-α in type II pneumocytes.[144]
4. Germination of conidia to form hyphae within the epithelial cells
5. Growth of hyphae by apical extension and escape from the epithelial cells

Within pulmonary vessels

6. Hyphael penetration of abluminal surfaces on the endothelial cells of pulmonary vessels
7. Endothelial cell damage by growing hyphae
8. Haematogenous dissemination of hyphal fragments

Within systemic blood vessels

9. Adherence of hyphal fragments to the luminal endothelial cell surface
10. Invasion of luminal endothelial cells
11. Endothelial cell damage
12. Extravascular invasion

These events are summarised in Figure 2.

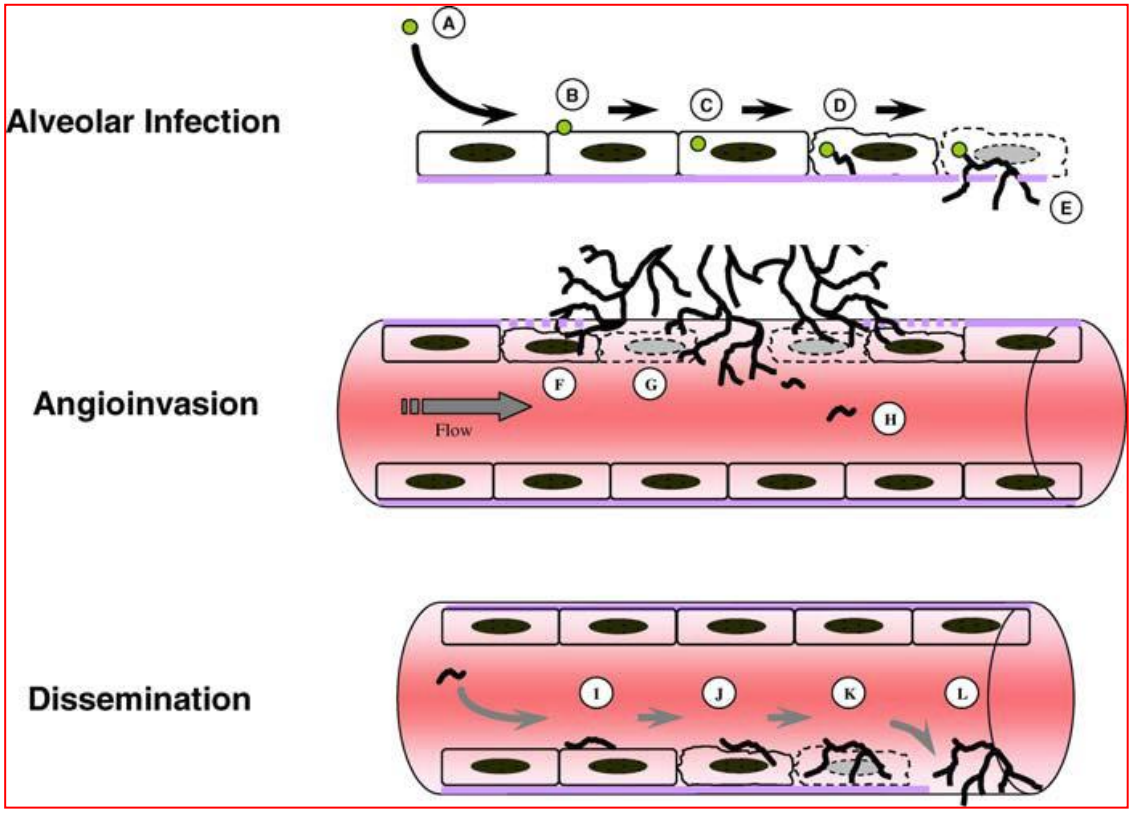


Figure 2 Model of *Aspergillus fumigatus* angioinvasion according to Filler et al [143]
IA occurs via the following steps: infection is initiated by the inhalation of small numbers of airborne conidia (A), which adhere to pulmonary epithelial cells (B), and are rapidly endocytosed (C). Within the epithelial cells, the conidia germinate to form hyphae (D), which grow by apical extension and escape from the epithelial cells (E). Next, emergent hyphae penetrate the abluminal surface of endothelial cells (F) and induce endothelial cell damage (G). Hyphal fragments disseminate hematogenously (H) and adhere to the luminal endothelial cell surface (I) before invading these cells (J). Luminal invasion results in endothelial cell damage (K) and extravascular invasion of deep organs (L).

Angioinvasion is a key feature of IA pathogenesis. As shown above there are 2 types of angioinvasion: pulmonary and systemic. Pulmonary angioinvasion causes endothelial injury, release of proinflammatory cytokines, tissue factor expression and activation of coagulation cascade.[145] Moreover *A.fumigatus* hyphae are potent inducers of platelet α-granules and lysosomal granules that leads to expression of membrane-bound (CD66 and CD62P) and soluble (RANTES, CD40L, DKK-1) inflammatory mediators.[146] Consequently these processes lead to thrombosis and thus reduced tissue perfusion of the infected lung tissue leading to coagulative necrosis and effectively sequestering the

infected area.[53] This is the hallmark of the histological and radiological appearance of IPA: a central zone of necrotic tissue heavily infected by hyphae and a peripheral zone of haemorrhage with notable absence of inflammatory infiltrate into the affected area (Figure 3).[147] The angioinvasion and subsequent coagulative necrosis prevents the recruitment of inflammatory cells to the site of infection. Likewise, the vasculopathy and the sequestration of the affected area results in poor tissue perfusion and subtherapeutic antifungal drug concentrations at the infected area, despite treatment with a sensitive antifungal agents.[148] The discrete nodule corresponds to the nodule surrounded by the ground-glass infiltrate ('halo sign') on computed tomography of the lungs.[149]

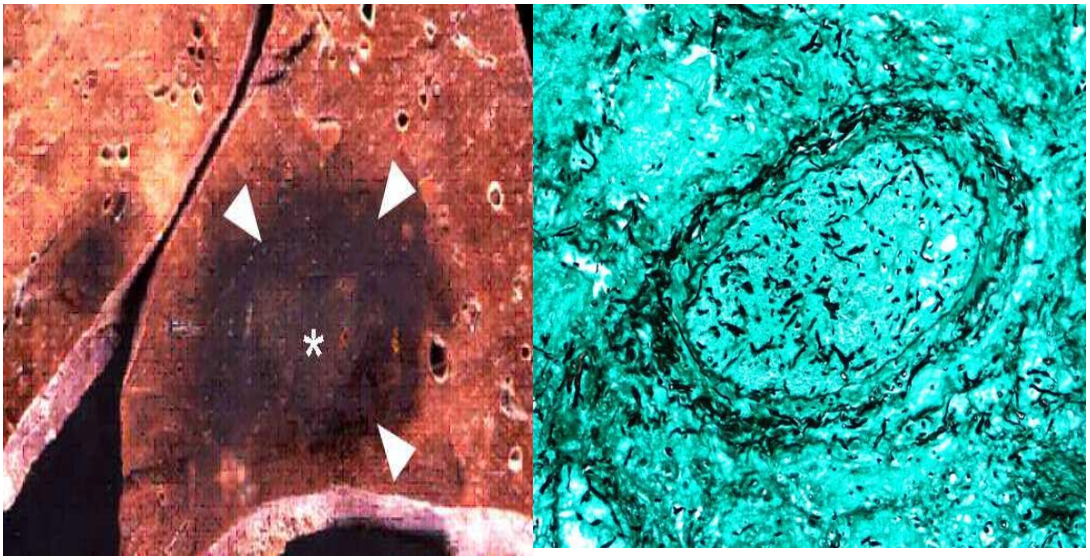


Figure 3: Histopathological appearances in angioinvasive aspergillosis. Post-mortem lung specimen (left) with two target lesions caused by angioinvasive aspergillosis. The larger focus in the lower lobe shows a central zone of necrosis (asterisk) and a surrounding "halo" of haemorrhage (arrowheads). High power image (x20) using Grocott stain shows fungal hyphae in the arterial lumen and surrounding lung (right).

Chamilos *et al* [77] found differences in the histopathological pattern of invasive pulmonary aspergillosis between neutropenic patients and non-neutropenic HSCT recipients with GVHD on immunosuppressive therapy. In the latter group there was more lung inflammation and less *aspergillus* burden in

contrast to the neutropenic patients who had less inflammation, and high *aspergillus* burden accompanied by hyphal angioinvasion and extensive coagulative tissue necrosis.

Systemic angioinvasion is less common but occurs in severely immunosuppressed and profoundly neutropenic patients.[143] During systemic haematogenous angioinvasion the hyphae invade the luminal endothelium in contrast to abluminal invasion in pulmonary angioinvasion. In an *in vitro* model Kamai et al demonstrated important differences between luminal and abluminal angioinvasion.[150] They found that luminal invasion was mediated by formation of endothelial pseudopods which engulfed the hyphae and that it caused greater endothelial cell damage but induced less expression of mRNA for E-selectin, TNF-α, IL-8, and tissue factor. The stronger proinflammatory and prothrombotic response to abluminal infection is probably designed to prevent haematogenous spread. The actual receptors that mediate invasion of endothelial cells are yet to be identified.

The formation of new blood vessels (angiogenesis) is a normal physiological response to inflammation and ischemia.[151-153] The consequent tissue hypoxia, proinflammatory cytokines and reactive oxygen intermediates (ROI) are important pro-angiogenesis signals.[94] The release of chemokines and cytokines (such as IL-8, TNF-α, HIF-1α) leads to the recruitment of neutrophils and macrophages to the lungs which release ROI such as hydrogen peroxide (H_2O_2) and others which upregulate NF-κB. The NF-κB induces the transcription of vascular endothelial growth factor (VEGF) and other pro-

angiogenic factors.[154-156] However, this normal physiological response is opposed by anti-angiogenic factors released by *Aspergillus spp*.

Gliotoxin is one of the most important known anti-angiogenic factors. It is released by *Aspergullus* species as a secondary metabolite synthesised under transcriptional regulation of *LaeA*, a transcription factor that controls secondary metabolism.[157] It downregulatates NF-κB directly and indirectly by reducing the concentration of H_2O_2.[158-160] Other antiangiogenic secondary metabolites have been identified such as fumagillin [161], pseurotin.[162]

1.3 Clinical features

The ubiquitous nature of *Aspergillus* species means that all body cavities are target areas for colonization and invasion. The upper and lower respiratory tracts are the most frequent sites where inhalation of *Aspergillus* conidia is the route of infection. In immunocompetent hosts, alveolar macrophages and neutrophils phagocytose and destroy the spores before they cause disease. In the immunocompromised host this primary defence is inadequate allowing the conidia to germinate into hyphae which then invade blood vessels leading to angioinvasive disease and haematogenous spread to other organs.[163,164] Unfortunately clinical features are non-specific by themselves however suggestive. In the upper respiratory tract the patient may experience facial swelling, epistaxis, proptosis, and cranial nerve abnormalities due to either direct orbital involvement or from cavernous sinus involvement (rhinocerebral aspergillosis) as well as bone erosion or ischemia of the palate.[32]

The lungs are commonest site of IA and patients commonly present with broad-spectrum antibiotic-resistant fever, which may be associated with cough, dyspnoea, pleuritic chest pain, or haemoptysis. Immune reconstitution inflammatory syndrome (IRIS) may occur in patients during neutrophil recovery presenting with clinical respiratory and radiological deterioration but showing microbiological response as evidenced by the rapid normalization of serum galactomannan.[165] Rapid recovery of neutrophils in patients with IPA may also be associated with pneumothorax or fatal haemoptysis.[166-168]

The second commonest site of IA is the central nervous system (CNS).[79,86,169] Patients presents with seizures, altered levels of consciousness with or without focal neurological signs. It is not possible on clinical or radiological grounds alone to distinguish cerebral aspergillosis from other causes of space occupying lesions such as toxoplasmosis, tuberculosis or even malignancy and biopsy is necessary to confirm the diagnosis. Cerebral aspergillosis may occur without pulmonary disease.[13] Gastrointestinal tract involvement manifests with abdominal pain, haematemesis, or melaena.[169] In disseminated disease skin lesions such as nodules which may be necrotic, crusted or echymotic with surrounding cellulitis or erythema may occur.[2,169] Other sites of involvement include kidneys, liver, spleen, adrenal glands, bone, pleura, heart, stomach, and large vessels.[13] Recipients of HSCT are more likely to develop disseminated disease.[7]

1.4 Prevention & Prophylaxis

The prevention of IA involves environmental infection control measures to reduce exposure to *Aspergillus spp* and other fungi as well as pharmacological prophylaxis during at risk periods.[170] There are two ways to provide environmental control: laminar air flow and high-efficiency particulate air (HEPA) filtration. Laminar air flow involves air filtration, reverse isolation, gut decontamination and non-absorbable antimicrobials as prophylaxis during the treatment of haematological malignancy.[171] It has been shown to be effective in reducing septicaemia and other types of infections in patients with leukaemia and aplastic anaemia undergoing HSCT.[172,173] The most important

component of laminar air flow is filtered air. Filtration with HEPA during periods of neutropenia provides effective prophylaxis against mould infection.[5,174,175] Potted plants, plant material, marijuana, and tobacco smoking are important environmental sources of *Aspergillus* spores and should be avoided.[171] However, pre-exposure to *Aspergillus* spp. is probably universal and such strategies can realise only a limited reduction in IA incidence. The role of a 'neutropenic diet', which excludes certain foods especially fresh vegetables and fruits, in preventing IA is unclear and currently there is no data on its efficacy but may have a role in the prevention of bacterial infections.[176,177]

Antifungal prophylaxis offers some promise especially among high risk patients. It is important to differentiate between primary and secondary prophylaxis and the changing practice landscape over time. Most of the data that exist in the efficacy of antifungal usage in this area relates to primary prevention and secondary prophylaxis will be dealt with separately. There are three classes of agents used for prophylaxis: polyenes, triazoles, and echinocandins.

1.4.1 Polyenes

Nystatin was the first antifungal agent discovered in 1951.[178] It was originally called Fungicidin and later Nystatin, New York State Department of Health, where its discoverers worked and was effective against oral mucosal candidiasis but was too toxic for systemic use.[179] Amphotericin B deoxycholate was the first systemic agent discovered in 1956, isolated from

Streptomyces nodosus.[180] It has striking structural similarity to nystatin (Figure 4) and similar antifungal activities but is less toxic and can be administered intravenously. Polyenes exert their antifungal activity primarily by binding ergosterol, the principal sterol in the cell membrane of fungi. This leads to the formation of transmembrane pores which leads to efflux of protons and cations, depolarization of the cell membrane and ultimately cell death.[181,182]

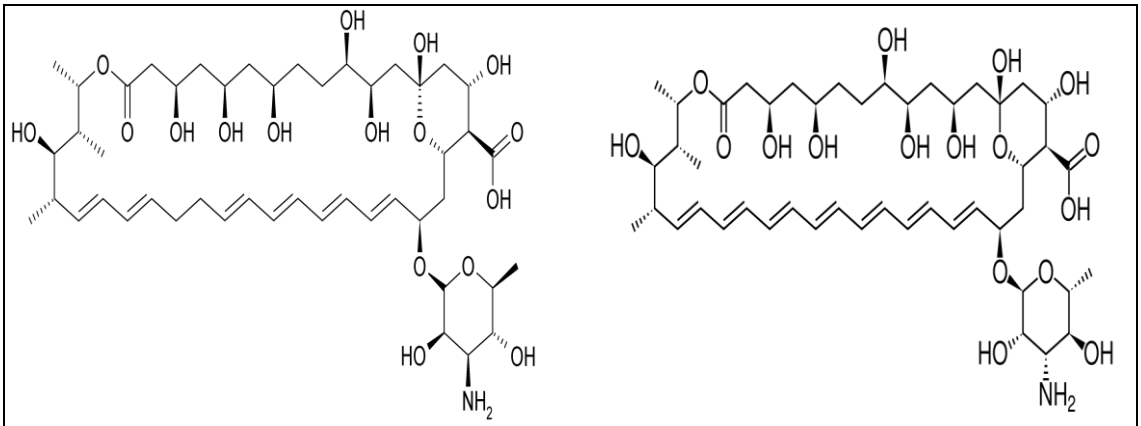


Figure 4: Structure of Nystatin (left) and Amphotericin B (right)

Lipid formulations of amphotericin B (L-AmB) with less nephrotoxicity were developed in the late 1980s. The evidence for the efficacy of Amphotericn B in preventing IA is conflicting. The trials using conventional amphotericin B versus placebo used low dose regimens of 0.1mg/kg/day and four such trials were conducted in the late 1980s and early 1990s. Two of the studies showed a reduced incidence and mortality associated with IA among allogeneic HSCT patients receiving amphotericin B compared to placebo.[183,184] However two

other studies showed no difference in IA incidence or survival benefit.[185,186] Four randomised trials were conducted using low dose L-AmB. The first two were safety studies involving allogeneic and autologous HSCT patients using 1mg/kg/day and 2mg/kg 3 times weekly vs placebo respectively. Although safety was demonstrated in both, no difference was seen in the incidence of proven IFD or requirement for use of antifungal therapy.[187,188] The third study was an open label randomised trial of 231 patients undergoing chemotherapy or autologous HSCT randomised to 50 mg Ambisome on alternate days or placebo. This study demonstrated a significant reduction in the incidence of IA: 4% compared to 20%.[189] The fouth study was non randomised study which compared amphotericin lipid complex (Abelcet) 2.5mg/kg 3 times weekly in AML and MDS patients undergoing induction chemotherapy with a historical control group that received Ambisome 3mg/kg 3 times weekly. No difference was seen in the incidence of documented fungal infection between the two groups.[190] Different dosing schedules were explored in two further studies. El-Cheikh *el at* evaluated weekly Ambisome at 7.5mg/kg in patients with acute GVHD on high dose prednisolone after reduced intensity transplantation while Cordonnier *et al* used 10mg/kg in 29 patients with acute leukaemia undergoing chemotherapy or myeloablative transplantation.[191,192] These doses were not well tolerated due to infusion-related toxicities and elevated serum creatinine.

Aerosolised administration of Amphotericin B has been used as a prophylactic strategy. Conventional Amphotericin B was neither efficacious in preventing IFD nor well tolerated.[193,194] Rijinders et al performed a randomised placebo-controlled trial of aerosolised liposomal Amphotericin on two consecutive days

per week in 271 patients with haematological malignancies with prolonged neutropenia.[195] The primary end point was development of proven/probable IPA using the 2002 EORTC/MSG definitions.[9] This strategy was both well tolerated and significantly reduced the incidence of IPA (14% among placebo v 4% amphotericin group; OR 0.26, P=0.005).

1.4.2 Triazoles

The principal agents in use in current haematology practice are fluconazole, itraconazole, posaconazole, and voriconazole. Triazoles act principally by inhibiting CYP450-dependent enzyme lanosterol 14α-demethylase which is necessary for the conversion of lanosterol to ergosterol.[171] Ergosterol is the predominant cell membrane sterol of most fungi. Table 8 summarises the evidence for the efficacy of triazoles as prophylactic agents in current haematological practice. The introduction of fluconazole in the late 1980s was a major advance. Not only did it significantly reduce IFD rate, mostly due to Candida spp,[196 197,198] but it also had long-term survival benefit when given for 75 days post-transplant.[198,199] At the time of these trials the dose used for prophylaxis was 400mg/day as this was thought to have some anti-mould activity. In fact fluconazole has no anti-mould activity and lower doses 50-200mg/day are also effective against candida.[200,201] Predictably, the widespread use of fluconazole prophylaxis prevented candidiasis but did not reduce mould infection, especially IA.

Itraconazole has good *in vitro* anti-mould activity and this was borne out in clinical trials in the setting of chemotherapy or HSCT.[202,203] However, itraconazole usage is hampered by two major issues- gastrointestinal side-effects and serious drug interactions. It is available orally as a capsule or cyclodextrin suspension and the former requires an acidic environment for absorption and generally poorly absorbed. In the setting of allogeneic HSCT its most serious drug interaction is with cyclophosphamide, where it was found to cause high bilirubin and creatinine due to differential inhibition of cytochrome P450 isoenzymes resulting in higher cyclophosphamide toxic metabolites.[204] Vinca alkaloid-induced neurotoxicity is also an important drug interaction with itraconazole as well as fluconazole and voriconazole.[205,206]

Posaconazole is the newest triazole prophylactic agent and is an analogue of itraconazole. Its efficacy has been shown in two large randomised trials in the context of patients with AML or MDS undergoing chemotherapy or patients with chronic GVHD post allograft.[207,208] Cornely et al randomised 602 AML or MDS patients with prolonged neutropenia in a multicentre non blinded study to receive either posaconazole 200mg tds (n=304) or fluconazole 400mg/d (n= 240), or itraconazole 200 mg bd (n= 58).[207] The study patients received prophylaxis with each cycle of chemotherapy until neutrophil recovery and complete remission (CR), breakthrough IFD (according to the original EORTC/MSG definitions[9]) or up to 12 weeks. The primary end point was the incidence of probable or proven IFD which occurred in 7 patients (2%) in the posaconazole arm and 25 patients (8%) in the fluconazole/itraconazole arm. IA was less in the posaconazole arm at 1% vs. 7% in the

fluconazole/itraconazole arm (P<0.001). It is worth noting that the definition of IA in this study relied heavily on galactomannan positivity which was not necessarily accompanied by radiological features, cases which would be unclassified under the current EORTC/MSG definition. Overall survival was significantly longer in the posaconazole arm.

In the setting of HSCT, Posaconazole has also been evaluated. There is limited data on its efficacy as primary prophylaxis as there is only one published study that evaluated this. Sanchez-Ortega *et al* compared 33 patients within 100 days of allograft to 16 itraconazole historical controls and showed 0% and 12% incidence of probable/proven IFD in the posaconazole and itraconazole groups respectively.[209] Overall survival and fungal free survival were also improved in the posaconazole group. The role of posaconazole prophylaxis in the context of extensive chronic GVHD was assessed by a double-blind randomised controlled trial of posaconazole compared to fluconazole in 600 patients by Ullmann *et al*.[208] The incidence of IFD and IA was lower in the posaconazole arm (2.4% and 1.0% respectively) compared to the fluconazole arm (7.5% and 5.9%). Overall survival was not significantly different between the two arms.

Voriconazole has recently been evaluated as a prophylactic agent in two large multicentre randomised trials in the context of neutropenia post allograft.[210,211] Wingard *et al* randomised 600 allograft patients to voriconazole or fluconazole.[210] The incidence of IFD, using the revised EORTC definitions,[10]

fungal-free survival, and overall survival were not significantly different between the two arms at 6 or 12 months post-transplant. The incidence of proven/probable IFD in this study was similar to the Ullmann *et al* study.[208] The number needed to treat (NNT) with voriconazole to prevent one IFD was 26 [212] compared to 27 with posaconazole.[213] A recently published multicentre European study, The IMPROVIT study randomised 503 allograft patients between voriconazole and itraconazole and found similar low rates of IFD in the both arms (1.3% voriconazole arm v 2.1% itraconazole arm) but voriconazole was better tolerated.[211] Gergis *et al* retrospectively reviewed their experience with voriconazole in 97 patients compared to itraconazole (N= 36) and fluconazole (N= 36) who had GVHD on 1mg/kg/d of prednisolone.[214] The incidence of IA in this study was 0% in the voriconazole group compared to 7% in the itraconazole/fluconazole group.

Voriconazole has also been evaluated in patients undergoing chemotherapy. Vehreschild *et al* randomised 25 AML patients undergoing induction chemotherapy between voriconazole and placebo.[215] The primary end-point for this study was the incidence of pulmonary infiltrates up to day 21 after start of chemotherapy. They found no infiltrates in the voriconazole arm compared to 33% in the placebo arm. In addition four cases of hepatosplenic candidiasis were also found in the placebo arm. The trial was prematurely terminated upon publication of the Cornely *el al* [207] study as the authors felt it was unethical to continue in the light of improved survival with posaconazole. Finally Mattiuzzi *et al* compared efficacy and safety of voriconazole to itraconazole, both given intravenously in 123 AML or MDS patients undergoing induction or salvage

chemotherapy.[216] The incidence of IFD was 0% in the voriconazole group compared to 4% in the itraconazole group (P= 0.17). There was significant toxicity in both arms. The cost implications and logistics of such an approach would be difficult to justify in clinical practice except where patients are unable to take oral agents.

Table 8: Triazole antifungal prophylaxis trials (Adapted from [179])

Author	Design	Population	Agents	Outcome (IFI, mortality)	Comments
Fluconazole					
Goodman[197] (1992)	RCT DB single centre *n* = 356	Autologous = allo geneic HSCT. Up to engraftment	Fluconazole 400 mg/d vs. Placebo	IFD 2·8% vs. 15·8% Mortality no significant difference	No survival benefit with fluconazole. Stopped on engraftment
Slavin[198] (1995)	RCT DB single centre *n* = 300	Allogeneic > autol ogous HSCT Up to day 75	Fluconazole 400 mg/d vs. placebo	IFD 7% vs. 18% Mortality significantly less at day 100	Fluconazole survival benefit persisted at 8 year follow-up (Marr[199], 2000)
MacMillan [201] (2002)	RCT single centre *n* = 253	Allogeneic (55%) and autologous (45%) Adult and paediatric patients	Fluconazole 400 mg vs. 200 mg/d	No difference in systemic IFD at day 50, 4% vs. 1% (*P* = 0·08)	Low incidence of IFD both arms
Menichetti [217] (1994)	RCT multicentre *n* = 820	Acute leukaemia, induction or reinduction	Fluconazole 150 mg/d vs. AmB 500 mg oral QID	No difference in IFD Fluconazole (2·6%) vs. AmB (2·5%)	
Winston [218] (1993)	RCT DB multicentre *n* = 257	Acute leukaemia anticipated ANC < 0·5 × 10^9/l for ≥7 d	Fluconazole 400 mg/d vs. placebo	No difference in IFD (4% vs. 8%) or mortality	
Rotstein [196] (1999)	RCT DB, multicentre *n* = 304	AML/autologous HSCT, intensive chemotherapy	Fluconazole 400 mg/d vs. placebo	Proven IFD17% vs. 3% (*P* < 0·001) No overall survival benefit	Most useful in cytarabine/anthra cycline for AML induction and autologous HSCT without growth factor support
Kern [219] (1998)	RCT multicentre *n* = 68	Relapsed and refractory AML	Fluconazole 400 mg/d vs. placebo	No difference IFD or survival	
Itraconazole					
Winston [202] (2003)	RCT DB multicentre *n* = 140	Allogeneic HSCT	Itraconazole 200 mg bd vs. fluconazole	Proven IFD 9% vs. 25% (*P* = 0·01). IA 4% vs. 12% (ns) Trend towards fewer fungal deaths with itraconazole (9% vs. 18%, *P* = 0·13, ns)	More GVHD in fluconazole arm Gastrointestinal intolerance 24% vs. 9%
Marr [203] 2004	RCT DB single centre *n* = 299	Allogeneic HSCT	Itraconazole 2·5 mg/kg tds vs. fluconazole 400 mg/d	Invasive mould infection 5% vs. 12% (*P* = 0·03) IFD 7% vs. 15% (*P* = 0·03) Mortality difference not significant	Discontinuation due to gastrointestinal intolerance 36% vs. 16% (*P* < 0·001)
Morgenstern [220] (1999)	RCT multicentre *n* = 445 (581 neutropeni c episodes)	Allogeneic, autologous HSCT and chemotherapy	2·5 mg/kg oral solution bd vs. fluconazole 50 mg/d	IFD one with itraconazole vs. six with fluconazole (*P* = 0·06)	Note: most infections in the low dose fluconazole arm were due to *Candida* spp
Nucci [221] (2000)	RCT DB two	Autologous HSCT,	Itraconazole capsules	IFD itraconazole 6% vs. 15% placebo (*P* = 0·03)	

	centres *n* = 210	haematological malignancy with expected duration neutropenia >7 d	100 mg bd vs. placebo		
Menichetti [222] (1999)	RCT DB multicentre *n* = 405	Autologous HSCT, haematological malignancy	Itraconazole oral solution 2·5 mg/kg bd vs. placebo	Proven and suspected IFD itraconazole (24%) vs. placebo (33%) (*P* = 0·35) No difference in mortality	
Harousseau [223] (2000)	RCT DB multicentre *n* = 557	Autologous HSCT, acute leukaemia	Itraconazole oral solution 2·5 mg/kg bd vs. AmB capsules 500 mg QID	No difference in proven IFD. Itraconazole (2·8%) vs. AmB (4·7%)	
Posaconazole					
Ullmann [208] (2007)	RCT DB multicentre *n* = 600	Allogeneic HSCT with ≥grade 2 or chronic extensive GVHD	Posaconazole 200 mg tds vs. Fluconazole 400 mg/d	Proven/probable IFD 7% vs. 14% Mortality difference not significant	Few patients with most severe form of GVHD (grade 4) at entry
Cornely [207] (2007)	RCT not blinded multicentre *n* = 602	AML/MDS with intensive chemotherapy	Posaconazole 200 mg tds (n= 304) vs. fluconazole 400 mg/d (*n* = 240) or itraconazole 200 mg bd (*n* = 58)	Proven/probable IFD 2% vs. 8% (*P* = 0·0009). Mortality15% vs. 22%*P* = 0·03 Serious adverse events possibly or probably related to treatment in 19 (6%) posaconazole group and 6 (2%) in the fluconazole or itraconazole group (*P* = 0·01)	Not powered to compare itraconazole. Many of cases diagnosed by positive serum galactomannan
Voriconazole					
Wingard [210] (2010)	RCT DB multicentre *n* = 600	Allogeneic HSCT (HLA matched in at least 5/6 loci)	Voriconazole 200 mg bd vs. fluconazole 400 mg daily	Fungal-free survival at day 180 was not different at 75% and 78% IFD not significantly different voriconazole (7·3%) vs. fluconazole (11·2%)	Patients were monitored with serum galactomannan tests twice weekly to day 60, then weekly
Marks [211] (2011)	RCT open-label multicentre *n* = 503	Allogeneic HSCT	Voriconazole 200 mg bd vs. itraconazole 200 mg bd	Proven/probable IFD 1.3% v 2.1%. OS similar. Vori better tolerated	Quite low number of cases in both arms

IFD, invasive fungal disease; HSCT, haematopoietic stem cell transplant; ANC, absolute neutrophil count; HLA, human leucocyte antigen; AML, acute myeloid leukaemia; MDS, myelodysplastic syndrome; ns, not significant; GVHD, graft versus host disease; IA, invasive aspergillosis; i.v., intravenous; AmB, amphotericin B, RCT, randomised controlled trial

1.4.3 Echinocandins

The echinocandins are a novel class of antifungals that work by inhibiting β-1,3-D-glucan synthase, the enzyme complex that catalyses the synthesis of β-1,3-D-glucan, an integral component of the fungal cell wall that provides rigidity, antigenicity and fungal virulence.[224] The uniqueness of the fungal cell wall and the specific action of the echinocandins may account for their safety and minimal drug interactions. They are large molecules and are only available parenterally due to their poor oral bioavailability.[225] Micafungin versus fluconazole has been studied for prophylaxis in allogeneic HSCT recipients in 2 randomised trials and Micafungin was found to be effective in invasive candida infection but without evidence of efficacy against IA.[226,227] Caspofungin has been shown to be as effective as itraconazole in patients undergoing chemotherapy.[228]

1.4.4 Secondary prophylaxis

Patients with previously documented evidence of IA who need further chemotherapy are given secondary prophylactic antifungal therapy during additional chemotherapy or transplantation to pre-empt relapse of IA. These patients are at high risk of infection relapse without secondary prophylaxis.[229] This issue has been addressed in 2 small non-randomised studies. Vehreschild et al evaluated 75 cases from the Multinational Case Registry of Secondary Antifungal Prophylaxis who received caspofungin (N= 47) or itraconazole (N= 28).[230] Patients treated with caspofungin were more likely to have uncontrolled underlying malignancy, incomplete response to previous

antifungal therapy and more likely to undergo allogeneic HSCT. Breakthrough IFD occurred in a third of the patients and overall survival was comparable between the groups. The second study was a prospective open label multicentre trial conducted by the European Group for Blood and Marrow Transplantation (EBMT) and evaluated the efficacy of voriconazole in 45 allograft recipients who had previous proven or probable IFD.[231] The 1-year cumulative incidence of IFD was 6.7%. Although 11 patients (24%) died within 12 months of transplant, only one was due to systemic fungal infection. The results of this study are much better than historical controls and argue strongly in favour of secondary prophylaxis with voriconazole.

1.4.5 Guidelines

The above evidence and those derived from meta-analysis support the use of antifungal prophylaxis in high risk patients and is recommended by guidelines on both sides of the Atlantic.[4,11,232] Table 9 summarises the updated European Conference on Infections in Leukaemia (ECIL-3) and the Infectious Disease Society of America (IDSA) consensus guidelines for antifungal prophylaxis in AI. The grading is based on the IDSA grading system.[233]

Table 9: Summary of the ECIL-3 and IDSA guidelines for prophylaxis against IA

	ECIL-3 grading [11]	**IDSA grading** [4]
Induction chemotherapy		
Fluconazole	C-I	-
Itraconazole	C-I	-
Posaconazole	A-I	A-1
Aerosolised L-AmB & fluconazole po	B-I	-
AmB and L-AmB	C1	-
Allogeneic HSCT, neutropenic phase		
Fluconazole	A-I	-
Itraconazole	B-I	-
Posaconazole	No data	-
Voriconazole	A-I (provisional)	-
Aerosolised L-AmB & fluconazole po	B-III	-
Micafungin	C-I	-
L-AmB	C-I	-
Allogeneic HSCT, GVHD phase		
Fluconazole	C-I	-
Itraconazole	B-I	B-I
Posaconazole	A-1	A-1
Voriconazole	A-I (provisional)	-

Despite the clinical trials discussed previously, the overall benefit, cost effectiveness and risks of primary prophylaxis remains a matter of debate.[234,235] In addition, mould-active azoles can cause serious drug interactions through their inhibition of the hepatic cytochrome P450 enzyme system as well as cause serious side effects such as hepatic toxicity. Moreover, there remains a serious concern about the effectiveness of these agents due to poor absorption which may be an important issue in patients

with severe mucositis or diarrhoea as well as gastrointestinal intolerance. Because of this, therapeutic drug monitoring is recommended.[4,11] The use of primary prophylaxis may also cause low *Aspergillus* antigenaemia leading to false negative serological tests such as galactomannan.[32] Local institutional fungal epidemiology and availability of timely diagnostic facilities are crucial. It is argued that for primary prophylaxis to be effective the background incidence need to be >5% since the number of patients needed to be treated to prevent one fungal infection would be >20, a figure suggested as the cost effective threshold.[236]

1.5 Treatment of IA

Currently, three different classes of antifungal agents are licensed for the treatment of IA: polyenes, azoles and echinocandins. How these drugs are used varies considerably in different clinical practice but can be broadly categorised as: empirical, pre-emptive and targeted therapy. This is largely because currently there are no reliable diagnostic tools sufficiently sensitive and specific enough for the diagnosis of IA in routine clinical practice. Empirical treatment is the initiation of antifungal therapy among high risk patients at first suspicion of IA.[237] In practice this usually means bacteria culture-negative neutropenic sepsis resistant to broad-spectrum antibiotics for 4-7 days but no specific radiological or laboratory evidence of IA. This fever-driven strategy was developed in the 1980s when there were no adequate diagnostic tools available.[238] However, fever has a low positive predictive value for diagnosing invasive fungal infection[239] and may miss cases in patients on high dose steroids or other immunosuppressive therapy (low negative predictive value).[238] Clearly this approach will lead to the treatment of a large proportion of patients who have no IA. It has been the standard of care widely used and endorsed by guidelines, although it has never been compared to placebo or other treatment strategies.[4,11,239-241]

The availability and the use of non-culture based methods of serological detection of cell components of *Aspergillus* such as galactomannan or β-D-glucan as well as modern CT scanning techniques has given rise to the term 'pre-emptive' therapy or the diagnostic-driven strategy. It relies on sensitive

diagnostic tools such as galactomannan, β-D-glucan or PCR, as well as CT scanning in high-risk patients to guide therapy before full blown disease develops. This approach has been shown to reduce anti-fungal usage to about 50% of the empirical approach [242] and has been recently shown to be non-inferior to empirical therapy in a randomised trial.[243] Targeted therapy is where pathogen identification has been achieved histologically or by culture from a sterile site.[237]

1.5.1 Empiric therapy

For many, Amphotericin B remains the gold standard for empiric therapy. However, it is a toxic antimicrobial with multiple infusion-related and other side-effects, including renal impairment which may be augmented by other nephrotoxic drugs.[6] Lipid preparations (liposomal amphotericin B [AmBisome], amphotericin B lipid complex [ABLC; Abelcet], ABCD [Amphocil or Amphotec], are less acutely toxic (and Ambisome may be marginally more effective) than amphotericin B, but are expensive and there remains evidence of less overt cumulative nephrotoxicity.[244-248] The efficacy of liposomal amphotericn B was based on a multicentre double blind randomised trial involving 687 patients randomised to either conventional or liposomal amphotericin B.[245] Liposomal amphotericin was as effective as conventional formulation for empiric therapy and was associated with fewer break-through infections, and less toxicity. All the lipid preparations preferentially distribute to the reticulo-endothelial system with relative sparing of the kidneys.[4] At the commonly prescribed dose of

3mg/kg/day AmBisome was as effective as 10mg/kg/day in the AmBiLoad trial but less nephrotoxic and had a trend towards better survival at 12 weeks.[249]

Walsh et al conducted a multicentre double-blind randomised trial involving 1095 patients who had persistent fever and neutropenia.[250] Using composite end points caspofungin was found to be as effective as and better tolerated than liposomal amphotericin B. A similar finding was observed in children.[251] The efficacy of Micafungin was assessed in a prospective, non-comparative trial involving 277 neutropenic adults in 87 Japanese centres.[252] Its efficacy was similar to previous empiric trials at 80.7% response rate. As a result of this trial, as well as another historical comparative study (Micafungin v caspofungin), [253] ECIL 3 accepted Micafungin as an empiric therapy.[11]

Voriconazole was compared with liposomal amphotericin B in a multicentre randomised trial involving 837 patients.[254] Using composite end point analysis to assess treatment efficacy, voriconazole failed the 10% non-inferiority cut off. Consequently it was not approved by the FDA for empiric therapy. Itraconazole is a possible alternative empiric agent if drug interactions are not an issue, but it has not been studied in allogeneic HSCT patients and blood levels should probably be monitored.[255,256]

1.5.2 Targeted therapy

This refers to treatment of probable and proven cases, but in some trials possible cases have been included.[238] Voriconazole is recommended worldwide as the drug of choice for first line therapy for IA based on a prospective, randomised trial with amphotericin B as the comparator. The trial involved 277 patients with proven or probable IA randomised to voriconazole or conventional amphotericin B. Patients treated with voriconazole had a significantly better response and survival rates (53% and 71% respectively) than the amphotericin B arm (32% and 58% respectively).[47] However, about a third of the enrolled patients in this study had no microbiological confirmation of IA (therefore possible IA).[257] Voriconazole is also considered the drug of choice for cerebral aspergillosis.[258] Particular clinical scenarios however, favour the use of non-azole-based therapy:

- prior exposure to mould active azoles
- drug interactions and contraindications
- moderate/severe hepatic and renal impairment
- presence of mixed fungal infections including zygomycetes as it has no activity against this group (mucorales).[238]

The AmBiLoad trial compared 3mg/Kg/d to 10mg/Kg/d for 14 days followed by 3mg/kg/d in a double-blind trial involving 201 cases where the primary outcome was complete or partial response at the end of the study. The response rates were similar between the two arms (50% v 46%) although the higher dose was more toxic and was associated with a trend to higher mortality

rate.[249] However, in this trial about 60% of the subjects were enrolled on the basis of suggestive radiological signs with no supporting mycological confirmation (possible disease).[257] As a result, there remains uncertainly about the comparability of this study to the voriconazole trial even though the reported response rates were similar.[47] Consequently, liposomal amphotericn B is considered an alternative drug of choice for first line therapy for IA.[4,241] Where there is mycological confirmation of *A. terreus* or where clinical suspicion is high, mould-active azoles should be used, as this organism is resistant to amphotericin B.[259]

There is a paucity of data on the use of echinocandins as primary therapy of IA. Two prospective non-comparative phase II studies exist. Caspofungin was evaluated as first-line monotherapy of IA in 61 proven/probable patients with haematological malignancies, undergoing autologous transplantation. The rate of complete/partial response was 33% and 12 week-survival rate was 53%.[260] Another study enrolled 24 proven/probable allogeneic HSCT patients and the 12 week response and survival rates were 33% and 50% respectively.[261] These studies are not directly comparable to previous studies as all patients in these caspofungin studies had mycologically documented disease and the majority had poor baseline characteristics (poor performance score, uncontrolled malignancy, and older age). Nonetheless, caspofungin cannot be recommended as first line therapy but does provide an option for those unable to receive voriconazole or liposomal amphotericin B.[11,238] Caspofungin is licensed for salvage therapy in patients with probable and proven IA refractory to or intolerant of other agents.

1.5.3 Salvage therapy

Information on the number or proportion of patients failing first line therapy is scarce. Where possible tissue diagnosis should be obtained and drug levels monitored in such cases. Therapeutic options include switching to a different class of drugs to that used in the first line; liposomal amphotericin B, itraconazole, posaconazole and caspofungin are all approved for salvage therapy.[4] Overall, up to 50% of those who failed first-line therapy can be salvaged.[238] Because of their novel properties there is substantial interest in combining echinocandins with either triazoles or polyenes to exploit inhibition of both cell wall and cell membrane properties.[32] Despite little supporting evidence, multiple antifungal regimens are increasingly used with augmented toxicity and substantially increased healthcare costs.[262-264] A multi-centre randomised controlled trial is underway comparing voriconazole plus placebo versus voriconazole plus anidulafungin.

1.6 Diagnosis

There is good evidence that delayed diagnosis of IA is associated with poorer outcome.[265,266] Very early therapy, when mould burden remains low and tissue necrosis minimal, offers the best chance of controlling infection. Although antifungals may prevent further loss of tissue and dissemination from the extending margins of an IA lesion, few antimicrobials penetrate into ischemic or necrotic tissue in adequate concentrations to exhibit an effect. IA usually presents as invasive pulmonary aspergillosis but infection may disseminate to brain, meninges, spleen, liver, skin and other sites. *Aspergilli* invade and block blood vessels producing infarction and extensive haemorrhagic necrosis. Neutrophils are the major defence and IA progresses particularly rapidly in neutropenic patients and those receiving corticosteroids.[267] Despite what may evolve to be a dramatic necrotizing pneumonia with dissemination, *aspergilli* are rarely isolated from clinical material pre-mortem: blood cultures are almost invariably negative and sputum culture has poor sensitivity and specificity.[265] The diagnostic yield of bronchoalveolar lavage in IPA is usually less than 25-30%. Culture is also slow. This, linked with a non-specific early presentation (typically pyrexia despite broad-spectrum antibacterials), delays diagnosis. A definitive diagnosis may be made by demonstrating invasion on histology, but this may be challenging in thrombocytopenic patients. In many centres a diagnosis of IA is usually inferred from characteristic changes on high definition CT scans. However, substantial tissue damage must have already occurred by the stage at which characteristic signs appear.[265,267] Antibody tests are of no value in

the diagnosis of IA as the at-risk patient population is usually incapable of mounting responses.[265] However, serological tests based on the detection of *Aspergillus* products are available and have been evaluated.[267]

The Invasive Fungal Infection Cooperative Group of the European Organisation for Research in Treatment of Cancer (EORTC) and the Mycology Study Group (MSG) of the National Institute of Allergy and Infectious Diseases formed a Consensus Committee in 1998 to develop standardised definitions for invasive fungal infections for clinical research. They proposed a consensus diagnostic criteria stratified into proven, probable, and possible invasive fungal infection based on clinical, host, and microbiological factors in 2002.[9] These have since been revised but the basic classification into the 3 levels of certainty remains unchanged (Appendix).[10]

The revised EORTC/MSG definition of proven aspergillosis requires histopathological proof and/or positive culture of a specimen from a normally sterile site. This is seldom possible in clinical practice as many of the patients who go on to have biopsy might have been on empirical treatment with antifungals for a few days before the biopsy. However, these would still be proven mould infections, if there is histological proof. The diagnosis of probable IA requires the presence of all 3 criteria: host factors (immunosuppressive state), clinical factors (radiological signs) and mycological evidence. The mycological evidence can either be direct; where there is cytological or culture evidence from sputum, broncho-alveolar lavage (BAL), bronchial brush, sinus aspirate samples, or it may be indirect; detection of

fungal antigen or cell wall constituents. Only galactomannan on serum, plasma, CSF and BAL and β-D-Glucan detected in serum are accepted as such evidence. Possible IA is defined by the presence of host and clinical factors but no mycological evidence. In practice proven and probable cases are combined into one category to define 'invasive aspergillosis'.[4] In the current revised definition patients with the appropriate host factors and mycological evidence who lack the clinical factor such as CT evidence of dense, well-defined lesions with or without a 'halo' sign, air crescent sign or cavity are not classified. In the old definition this was defined as possible IA.

1.6.1 Computed Tomography (CT)

The typical imaging signs of invasive pulmonary aspergillosis (IPA) have been established in studies which compared the relationship between histopathological features and corresponding appearances on CT.[268-270] On macroscopic histological sections, there are foci of necrotising pneumonia with zones of haemorrhage and acute and granulomatous inflammation (Figure 3).[271] This corresponds radiologically, on axial CT scanning, to a focus of consolidation surrounded by a zone (the so-called 'halo') of ground-glass opacification (Figure 5).[149,272] The halo sign is the first distinctive radiological feature of IPA. In previous studies, it has been suggested that the halo sign is specific for IPA.[149] However, biopsy series have shown that a diagnosis other than IPA is made in around 50-60% of patients even when there is a high clinical and/or radiological pre-test probability of pulmonary aspergillus infection.[268,270,273] Moreover, it is also clear that the halo sign is not sensitive

and, because the CT findings reflect the end-result of small vessel invasion and infarction, it seems likely that the halo sign occurs relatively late in the course of IPA.

Figure 5: CT “halo” sign of angioinvasive aspergillosis in a neutropaenic patient. Axial image through the upper zones in a patient with MDS transformed to AML. CT through the upper zones demonstrates multiple rounded nodules; the dominant right upper nodule and the lesion in the left upper lobe (arrows) both have surrounding halo of ground-glass opacification.

Whilst the typical CT findings have been established there remains uncertainty about the spectrum of CT findings in patients with IPA. The problem is that biopsy confirmation is seldom available. Thus, the range and prevalence of CT features, other than the classical (i.e. foci of consolidation with halos of ground-glass opacification) is unknown. Factors that might also influence the CT appearance of IPA (for instance, the use of concurrent/prophylactic antifungal chemotherapy, the severity of neutropenia, the underlying disease, etc) are

also unknown. During the first week following diagnosis, there is a tendency for the number and size of focal lesions to increase, irrespective of treatment.[274,275] Greene et al found that the presence of a halo sign was associated not only with an improved response to antifungal drugs but also with improved survival at 12 weeks.[149]

The second CT feature of IPA is the air crescent sign which, is a semilunar cavitatory lesion within a macronodule (Figure 6).[276] This generally coincides with neutrophil recovery.[149] There is an inverse relationship between the halo sign and the air crescent sign in the pattern of appearance from diagnosis.[274] Overt cavitation occurs later in the course of infection but its presence is less specific than either the halo or the air crescent signs.[149] The EORTC/MSG recognizes well-circumscribed lesion(s) with or without a halo sign, air crescent sign and cavity as evidence compatible with IPA.[10]

Another aspect of the CT diagnosis, which potentially warrants investigation, is the pattern of contrast enhancement. The background to this is the knowledge that vascular lesions (specifically, malignant lung nodules) demonstrate greater enhancement following the injection of intravenous contrast.[277] There is paucity of data among haemato-oncology patients and in particular how IPA may behave in such patients. We hypothesise that, because foci of IPA represent regions of infarcted lung, the *absence* of enhancement following the injection of intravenous contrast might identify areas of IPA and that this might also be of value in the differentiation from other inflammatory or infective pathologies in which contrast enhancement would be the expected finding.

Figure 6: Air crescents and cavitation in angioinvasive aspergillosis.

1.6.2 Tissue diagnosis-biopsy

Imaging appearances of the chest or any other suspected area of IA are non-specific.[268,270,273] However, the potential difficulties associated with surgical biopsy in patients with poor performance status, co-morbidities and, not infrequently, impaired clotting remains a real challenge. As such there is a paucity of data in the literature about the diagnostic utility of biopsy in haemato-oncology clinical practice. The limited published data in this area are small retrospective studies.[268,273,278,279] They suggest that the biopsy is safe and useful but the small size of these studies, often spanning many years, indicates that this diagnostic tool is underutilised in clinical practice and there may be significant selection bias. The current techniques for obtaining tissue biopsy include bronchoalveolar lavage, transthoracic percutaneous needle

aspiration (often CT-guided), open biopsy, and video assisted thoracoscopic surgery (VATS).

Less invasive techniques, such as VATS for surgical sampling of small intrapulmonary nodules hold promise but the issue of a false-negative biopsy results for nodules which are impalpable and/or not readily visible remains a crucial issue.[280] Methods for assisting the pre-operative localisation of small lung nodules, including the image-guided placement of a hook-wire [281,282] or platinum microcoils [283] have been tested. The easiest and safest appears to be the methylene blue technique of pre-operatively injecting methylene blue dye into the area under CT-guidance, which acts as a guide to the surgeon (surgical track) using a thin calibre needle. Data on the value of methylene blue 'tattooing' of small intrapulmonary nodules prior to the thoracoscopic resection are convincing and possibly presents a safe and accurate technique for localisation of pulmonary nodules.[284-286] However, their utility in diagnosis of IPA in haemato-oncology patients is unknown.

The typical histopathological features have been described and can be divided into two patterns.[77,147] The first pattern, usually seen in neutropenic patients, consists of a discrete nodule with central and peripheral zones (Figure 3). The central zone is the area of coagulative necrosis, invading hyphae, and vascular thrombosis surrounded by peripheral zone of haemorrhage. There is notable absence of inflammatory infiltration. The discrete nodule forms the basis of the halo sign. As patients begin to recover their neutrophils or in those with GVHD a different pattern emerges. This second pattern is described as fused lobular

consolidation, a broncho-pneumonic picture characterised by acute inflammatory exudates. It is thought that with neutrophil recovery the discrete nodule undergoes liquefaction necrosis leading to cavitation at the periphery of the persisting central zone of coagulative necrosis. This forms the basis of the radiological 'air crescent sign'.[149]

1.6.3 Galactomannan

The *Aspergillus* cell wall is complex meshwork of cross-linked polymers, mainly β glucans, chitin and galactomannan, which forms an interface between the fungus and its environment.[106] Galactomannan (GM) is a polysaccharide component of mannoproteins in the outer cell wall. GM belongs to a family of molecules called galactofurannose (Gal-f) antigens.[287] These antigens contain galactofuranose residues that react with the rat IgM monoclonal antibody (EB-A2) used in the enzyme immunoassay (EIA). The EB-A2 is directed against tetra (1→5)-β-D-galactofuranoside to both capture and detect Gal-f and has been applied widely for the diagnosis of IA in immunocompromised patients.[288-291] The Gal-f residue is also present in fungal glycoproteins including phospholipase C and phytase.[287,292] How these antigens are released by *Aspergillus* during infection and in what form they circulate in blood remains unclear.

The approved indication for the test is in monitoring *Aspergillus spp* antigenaemia in high risk haemato-oncology patients. The results are expressed as an index relative to the mean optical (OD) density of the

threshold controls- ‘galactomannan index’ (GMI) (GMI= OD of sample/mean OD of the threshold control samples).[289,291] The test was first commercially available in Europe in the mid-1990s when a GMI cut-off of ≥1.5 was considered positive and <1.0 as negative and indices of 1.0-1.5 as indeterminate. However, in the United States a cut-off of 0.5 was considered positive as this was the accepted value by US Food and Drug Administration when the test was approved for diagnostic purposes in 2003.[291,293] As a result different centres have adopted different GMI cut-offs to maximise sensitivity in their populations and undoubtedly this may account for some of the variation in the test performances.[290,291,294,295] Different studies have validated the cut-off of 0.5 and this has been shown to have a better performance among high risk haematology patients and is currently the internationally accepted standard.[267,290,291,296]

The performance of serum GM as a screening tool for high risk patients has been assessed in several studies which have demonstrated a specificity of greater than 85% consistently but varying sensitivities of between 29-100%.[294] In a meta-analysis by Pfeiffer et al on 27 studies from 1966 to 2005, the mean GM sensitivity was 71% (95% CI: 68-74%) and specificity was 89% (95% CI: 88-90%) for proven cases but there was a significant heterogeneity among different patient groups.[295] To increase the accuracy of the test it is suggested that it should be done twice weekly and that positive results should be retested.[289] The sensitivity of the test is reduced in patients on mould-active primary prophylaxis due to reduced antigenaemia. Marr et al report a

sensitivity of 52% in patients receiving mould active antifungal prophylaxis compared 89% in those with no mould active agents.[297]

One of the biggest draw backs of the test is false positivity. This is in part due to shared epitopes found on various products including foodstuffs, bacterial lipoglycans (eg *Bifidobacterium* spp.) and other fungi such as *Penicillium* spp. Table 10 lists the causes of false positive results. Piperacillin-tazobactam (Tazocin) and amoxicillin-clavulate or other β-lactam producing antibacterials are fermentation products of *Penicillium* spp and contamination with cell wall components of the *Penicillium* spp during production is the greatest problem in clinical practice and false positive results may occur up to 5 days after stopping antibiotic treatment.[298] Intravenous fluids containing gluconate, such as Plasmalyte, have also been found to cause false positive results as gluconate is also produced by fermentation in mould cultures.[299,300]

Table 10: Causes of false-positive galactomannan test (modified from [289])

False positivity caused by GM contamination	Cross-reactivity caused by similar cell wall GM
Antibiotics Piperacillin-tazobactam (Tazocin) B-Lactam antibiotics eg amoxicillin-clavulanate	Penicillum spp. including P. marneffei
Plasmalyte (sodium gluconate) or any other intravenous fluid/nutrition containing sodium gluconate	Histoplasma capsulatum
Possibly cotton, cardboard and soybean protein	Geotrichum
	Neosartoria
	Possibly Paecilomyces, Alternaria, Trychophyton, Botrytis, Wallemia, Cladosporium, Bifidobacterium

Although the Platelia *Aspergillus* test is standardised for serum, testing for the presence of GM using other body fluids such as bronchoalvelolar lavage (BAL) and cerebro-spinal fluid (CSF) is very promising. GM detection in BAL appears to be more sensitive than serum in patients with IPA [289,301-303] and is accepted EORTC/MSG criteria for the diagnosis of probable IA [10]. Similarly GM on CSF samples from patients with CNS aspergillosis may be useful and may result in earlier diagnosis.[304-306] However, the use of GM testing on CSF is at best investigational and has not been validated in any large scale study. Urine has been studied, mainly in small retrospective or case studies, using heterogenous methods for the detection of the galactomananan antigen.[307]

1.6.4 β-D-Glucan

β-1,3-D-Glucan (BG) constitutes a major carbohydrate fraction of cell wall (60%). It is present in most fungi with the notable exception of *Cryptococcus neoformans* and *Zygomycetes* [308] as well as some bacteria and plants.[309] BG detection, therefore, represents a 'pan-fungal' diagnostic test.[266,267,310] The BG testing is based on the ability of (1→3)-β-D-Glucan to activate the *Limulus (*amoebocyte of the horseshoe crab) coagulation pathway.[266] This *Limulus* reaction was first used to detect endotoxin which works via factor C, a protein serine zymogene (Figure 7).

The Fungitell test (Associates of Cape Cod, Falmouth, MA), developed using amoebocyte lysate fractions of the American horseshoe crab, and was licensed by the FDA in 2004 following clinical validation by Odabasi et al.[310] There is limited experience of performance of BG testing in IA patients but there is a suggestion that it may be as good as or even better than GM in some cases.[311,312] It is of course important to note that BG is not specific for IA and that candida and non-aspergillus mould infections, with the exception of zygomycosis and and cryptococcus may give positive results.[309] In addition false positive results may be caused by surgical gauze, intravenous immunoglobulin or albumin preparations, cellulose membranes in haemodialysis, and environmental glucan (fabrics, organic matter, air, settled dust).[309]

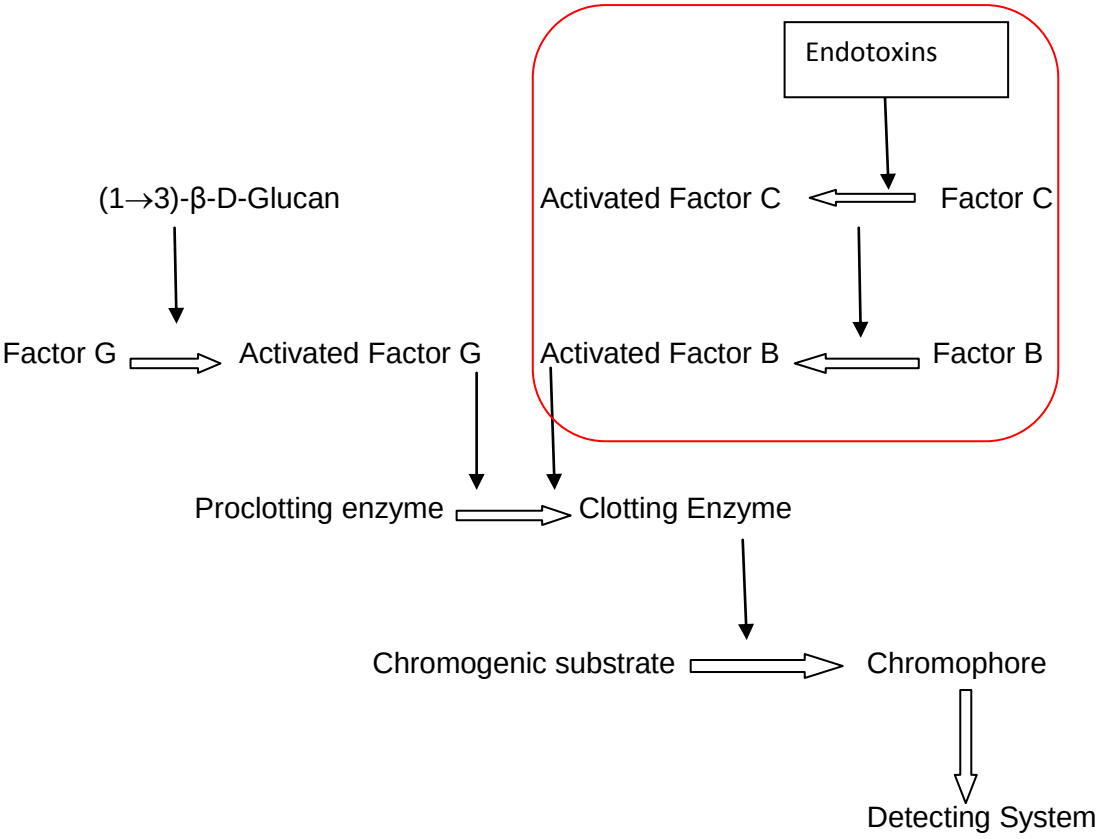


Figure 7: Mechanism of detection of (1→3)-β-D-Glucan by the use of horseshoe crab lysate. The enzymatic actions of endotoxin (in the box) are neutralised during the testing

1.6.5 Lateral Flow Device (LFD)

There is considerable interest in the search for newer, simpler and more reliable tests which can be used in clinical practice to accurately diagnose IA. The LFD is the latest attempt to fulfil these aspirations. It is based on an immunochromatographic device (similar to the home pregnancy test kit in design) incorporating a mouse IgG monoclonal antibody, JF5 which binds to an extracellular glycoprotein epitope constitutively secreted by growing *Aspergillus* spp hyphal tips.[313] The device has a release pad containing JF5 conjugated to colloidal gold particles which serves as the detection agent, a

porous nitrocellulose membrane, and a test line consisting of a JF5 line and a control line (commercial rabbit anti-mouse Ig). Serum samples are added to the release pad and which pass along the porous membrane by capillary action and then binds the JF5 antibody if the antigen is present. The bound antigen-antibody-gold complex appears as a red line and its intensity is proportional to the antigen concentration. The results are available in 10-15 minutes. Wiederhold et al have tested the performance of the LFD against GM and BG in a guinea pig IPA model and found the device positive earlier than GM or BG.[314] However, there is limited experience on clinical samples. Further validation using clinical samples with associated clinical information would be required before this test can be used in clinical practice.

1.6.6 PCR

Diagnosing IA by PCR in which *Aspergillus*-specific fungal genes, is likely to emerge as the most practical, sensitive and useful diagnostic approach.[266,315-319] Automated extraction methods are required to reduce contamination and make testing protocols feasible for routine diagnostic laboratories. Amplicon detection methods also vary; real-time detection maintains a low risk of contamination and reduces detection time. Various testing schedules, using different specimen types with single species and/or 'pan-fungal' PCRs, have been evaluated. These tests may be rapid (particularly with automated extraction), sensitive (capable of detecting 1-10fg/mL of DNA) and can be applied to various sample types. However, the lack of standardization makes PCR currently investigational.[4,32] In particular, there is lack of consensus on

the optimum specimen type (ie whole blood *versus* plasma or serum) which impacts on the method for DNA extraction, as well as the sampling schedule (timing and frequency of sampling). As such PCR has not yet been accepted as a diagnostic tool by the current EORTC/MSG.[10].

In an attempt to address the lack of standardised approach, a consensus protocol has been proposed comparing 2 primer sets (28S and 18S) on 3 platforms on a panel of 8 negative and 8 spiked samples.[320]. It was found that the 2Asp primers (targeting 28S gene) had higher sensitivity, specificity, and positive predictive value than the 4Asp (targeting 18S gene) irrespective of the platform. This initial effort has provided the stimulus for greater international collaboration to develop a consensus protocol.

1.7 Study objectives

1. To document the true ‘real world’ incidence and outcome of IFD in patients undergoing HSCT, high dose chemotherapy, or immunosuppressive therapy with expected neutropenic period of 10 days or more using all approved EORTC/MSG diagnostic tools;
2. To establish clinical utility of current diagnostic tests;
3. To assess the role of cytokines in the diagnosis and prognosis of IA;
4. To establish the prevalence, spectrum, natural history and prognostic value of CT appearances in patients with invasive pulmonary aspergillosis;
5. To assess the value and feasibility of surgical biopsy.

Chapter 2 Methods

2 Methods

2.1 Study design

This was a single centre prospective cohort study designed to evaluate the incidence and risk factors of IA in adult haemato-oncology patients undergoing HSCT or chemotherapy likely to render them neutropenic for 10 days or more. Figure 8 shows the schema of the study design.

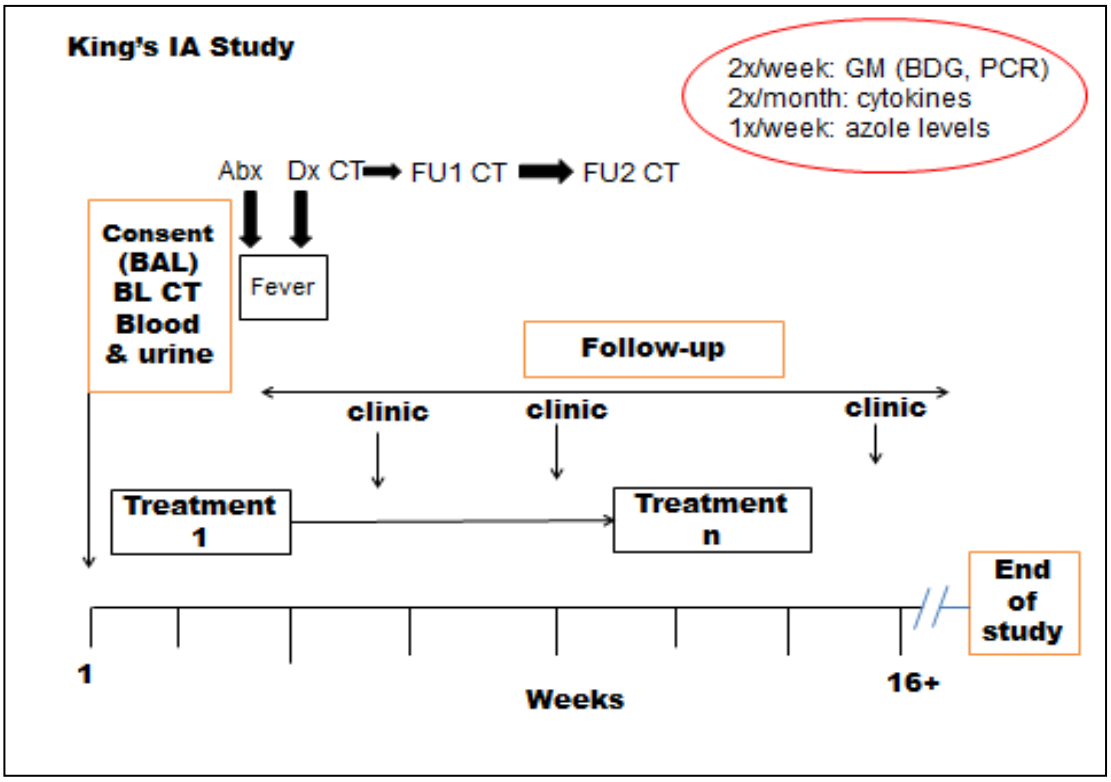


Figure 8: Study outline (not to scale). Each study patient had baseline CT, sera and urine taken for GM (±BDG). Broncho-alveolar lavage (BAL) was offered to transplant patients only. Mycology surveillance tests was done twice weekly during study period while receiving one or more cycles of treatment (allogeneic or autologous transplantation, chemotherapy or immunosuppressive therapy) until full haematological recovery. Antibiotic (Abx)-resistant fever was investigated with diagnostic (Dx scan) and follow-up (FU) 1 and 2 10-14 days and 28 days respectively after abnormal Dx scan.

All transplant patients were followed up for at least four months from transplant if they had no post-transplant complications such as GVHD. However, in the event of such complications they were followed up until this was completely resolved or they died or until the end of the study period. Similarly chemotherapy patients were followed up for at least four months after their last course of chemotherapy in the absence of any complications. Chemotherapy patients who then went onto transplant were followed up as per the transplant patients. In the event of death, the cause of death was ascertained in the light of all relevant clinical evidence and whenever possible a hospital post-mortem was performed.

2.2 Study procedures

After admission into the study each patient had:

- Clinical assessment (history, examination, weight, height, Karnofsky score) at admission and further evaluation (fever, presence of GVHD, respiratory symptoms) three times weekly during inpatient admission and during outpatient visits. A full drug history was also taken, in particular antifungal drugs used: duration and reason for starting and stopping.
- Blood tests (FBC, U&Es, liver function tests [LFT], Mg, CRP) at admission and three times a week during admission and outpatient visits.
- Cytokine profile at baseline and fortnightly during inpatient admission.

- Mycology (galactomannan, β-D-Glucan, PCR, lateral flow device [LFD]) at admission and twice weekly during inpatient admission and clinic visits.
- Virology (allogeneic transplant patients only): CMV, EBV and adenovirus at admission and twice weekly during first three months and once a week thereafter.
- Thoracic CT scanning

  All patients had a baseline non-contrast enhanced, low-dose scan. In addition, further scans were done:

  1. 'Diagnostic': contrast-enhanced thin section scans for patients who developed neutropenic sepsis unresponsive to second-line antibiotics (meropenem and vancomycin) for ≥48h with no positive microbiological cultures

  2. 'Follow-up': low dose non-contrast scans (x2) at two and four weeks post 'diagnostic' scan if the diagnostic scan had any abnormalities. This was to assess response to antifungal therapy.

- Bronchoscopy

  This was optional for transplant patients (both allogeneic and autologous) before the initiation of the conditioning chemotherapy. The samples collected were sent for routine bacteriology, virology, and biochemistry tests as well as galactomannan, β-D-glucan, and PCR. Additional samples were sent for cytokine profiling (luminex).

- Triazole trough levels weekly. The first levels were taken after at least a week of initiation of the azole.

- CXR weekly as part of routine clinical care
- Lung biopsy**.** Wherever possible neutropenic patients with sepsis and lung lesions had biopsies by video assisted thoracoscopic surgery (VATS) or open biopsy. Coagulation abnormalities were corrected before the procedure and platelets >50 $x10^9$/l on the day of the procedure.

2.3 Patients

Two hundred and three patients were recruited from King's College Hospital into the study between December 2008 and May 2010. The inclusion criteria were:

1. All adult (18 years and over) haemato-oncology patients admitted for transplant or chemotherapy likely to render them neutropenic (<0.5$x10^9$/L) for 10 days or more during treatment and
2. Able to give informed consent.

This includes the following patient groups:

- Allogeneic HSCT
- Autologous HSCT
- MDS
- AML
- Aplastic anaemia
- Salvage lymphoma chemotherapy such as CAIP, Hyper CVAD

The exclusion criteria were:

1. Children under the age of 18y
2. Refusal or inability to give informed consent

The patient characteristics are shown in Table 11. Primary treatment refers to the treatment that patients were admitted for at the time of enrolment into the study.

Table 11: Patient characteristics

N= 203	**characteristics**
Age (median, range) years	54 (19-73)
M/F	123/80
Undergoing diagnosis N (%)	
AML	55 (27)
MDS	26 (13)
MDS/MPD	3 (2)
MPD	4 (2)
Aplastic anaemia	19 (9)
CML	3 (2)
ALL	7 (3)
NHL	29 (14)
Hodgkin lymphoma	8 (4)
Multiple myeloma	45 (22)
Others*	4 (2)
Primary treatment N (%)	
Allograft	99 (49)
Autograft	66 (32)
Chemotherapy alone	28 (14)
IST	11 (5)
Median follow-up (range) days	19 (12-834)

*Others include CLL (1), plasma cell leukaemia (1), Blastic plasmacytoid dendritic cell neoplasm (1), and common variable immunodeficiency (1)

All patients received antifungal prophylaxis according to the existing King's protocol (Table 12). The high risk group included patients undergoing allogeneic HSCT, AML induction/consolidation, salvage lymphoma chemotherapy, Alemtuzumab, purine analogues, and high dose steroids

therapy. Autologous HSCT and outpatient chemotherapy unlikely to cause prolonged neutropenia were considered low risk in this protocol. The study was approved by the King's ethics committee and conducted in accordance with the Helsinki protocol (2008 revision). It was registered with the ClinicalTrials.gov (NCT00816088).

Table 12: KCH Adult Haemato-Oncology Antifungal Guidelines 2007

A. Prophylaxis		
	Primary	Secondary
Low risk	Fluconazole 200mg od po	Voriconazole 200mg bd Posaconazole 200mg tds Ambisome 2mg/kg M/W/F Caspofungin 70→50mg od
High risk	Itraconazole suspension 200mg bd If having vinca alkaloids: Ambisome 2mg/kg M/W/F Caspofungin 70→50mg od	

B. Therapy	
Empiric	• Ambisome 1→3mg/kg od • Casopfungin 70→50mg od
Proven/probable	• Ambisome 3mg/kg od • Voriconazole • Caspofungin 70→50mg od
Salvage	• Caspofungin 70→50mg od • Posaconazole • Flucytosine
Combination	Combined haematology and microbiology consultant decision

2.4 Diagnosis of IA

The diagnosis of IA was based on the current EORTC/MSG criteria.[10] On the basis of these criteria IA may be proven, probable or possible:

1. **Proven invasive aspergillosis**

Recovery of a mould by culture from a sample obtained by a sterile procedure from a normally sterile and clinically or radiologically abnormal site consistent with an infectious disease process, excluding BAL, cranial sinus cavity, and urine

OR

Histopathologic, cytopathologic, or direct microscopic examination of a needle aspiration or biopsy specimen showing hyphal forms with evidence of associated tissue damage. However, in the absence of a positive culture, proven IA cannot be made and such cases would be called proven mould infection.

2. **Probable invasive aspergillosis**

This was defined by at least one host factor + one clinical criterion + one mycological criterion

3. **Possible invasive aspergillosis**

Defined by one host factor and one clinical criterion but no mycological criterion

Host factors

1. Recent history of neutropenia (<0.5 x 10^9/L for >10 days) temporally related to the onset of fungal disease or ongoing neutropenia

2. Receipt of an allogeneic stem cell transplant

3. Prolonged use of corticosteroids (excluding patients with ABPA) at an average minimum dose of 0.3 mg/kg/day prednisone equivalent for >3 weeks

4. Treatment with other recognized T-cell immune suppressants such as ciclosporin, TNF-α blockers, specific monoclonal antibodies alemtuzumab, nucleoside analogues during the past 90 days

5. Inherited severe immunodeficiency (eg, chronic granulomatous disease, severe combined immunodeficiency).

All study patients fulfilled one or more of these criteria.

Clinical criteria

Must be consistent with the mycological findings, if any, temporally related to current episode and other potential causes must have been eliminated.

Lower respiratory tract fungal disease

The presence of one of the following 3 signs on CT:

- Dense, well-circumscribed lesion(s) with or without a halo sign
- Air crescent sign
- Cavity

Tracheobronchitis

Tracheobronchial ulceration, nodule, pseudo-membrane, plaque or eschar seen on bronchoscopic analysis.

Sinonasal infection

Imaging showing sinusitis, plus at least one of the following:

- Acute localized pain (including pain radiating to the eye);
- Nasal ulcer with black eschar;
- Extension from the paranasal sinus across bony barriers, including into the orbit.

CNS infection

At least one of the following:

- Focal lesions on imaging;
- Meningeal enhancement on MRI or CT.

Mycological criteria

This may be direct or indirect.

1. **Direct** demonstration of mould in sputum, BAL, bronchial brush, or sinus aspirate samples by the presence of fungal elements or recovery by culture of a mould.
2. **Indirect** serological evidence by detection of antigen or cell wall constituents such as galactomannan antigen EIA in plasma, serum, BAL, CSF or β-D-Glucan detected in serum.

Patients who did not satisfy the above categorisation into proven, probable or possible were classified into:

1. **No evidence**- no radiological or any other clinical abnormality, and no mycological evidence

2. mycological evidence but no clinical abnormality

3. abnormal radiology other than EORTC definition + mycological evidence

4. abnormal radiology other than EORTC definition without mycological evidence

scenarios 2-4 were considered 'not classified'.

2.5 Response to antifungal therapy

The EORTC/MSG consensus criteria were used to assess response at two and four weeks after initiation of antifungal therapy (Table 13).[321]

Table 13: Responses to antifungal therapy in patients with invasive mould disease[321]

Outcome/ response	Criteria
Success	
Complete response	Survival and resolution of all attributable symptoms and signs of disease; plus
	Resolution of radiological lesion(s); persistence of only a scar or postoperative changes can be equated with a complete radiological response; plus
	Documented clearance of infected sites that are accessible to repeated sampling (e.g., mould disease involving the palate, sinuses, or cutaneous lesions)
Partial response	Survival and improvement of attributable symptoms and signs of disease; plus
	At least 25% reduction in diameter of radiological lesion (s); plus
	Documented clearance of infected sites that are accessible to repeated sampling (e.g., mould disease involving the palate, sinuses, or cutaneous lesions)
	In cases of radiological stabilization (defined as a 0%–25% reduction in the diameter of the lesion), resolution of all attributable symptoms and signs of fungal disease can be equated with a partial response
	In cases of radiological stabilization, biopsy of an infected site (e.g., lung biopsy) showing no evidence of hyphae and negative culture results can be equated with a partial response
Failure	
Stable response	Survival and minor or no improvement in attributable symptoms and signs of disease; plus
	Radiological stabilization (defined as a 0%–25% reduction in the diameter of the lesion); or
	Persistent isolation of mould or histological presence of invasive hyphae in infected sites
Progression of disease	Worsening clinical symptoms or signs of disease; plus
	New sites of disease or radiological worsening of preexisting lesions; or
	Persistent isolation of mould species from infected sites
Death	Death during the pre-specified period of evaluation regardless of attribution

2.6 Laboratory methods

2.6.1 Galactomannan (Platelia™ *Aspergillus* EIA, Bio-Rad)

This test was routinely available at King's and was performed according to the manufacturer's instructions. Briefly, 300µL of serum was added to 100µL of 4% EDTA treatment solution, heated at 100 ºC for 3 min, and centrifuged at 10,000 *g* for 10 min. Fifty microlitres of the supernatant was added to 50 µL of conjugate (anti-GM monoclonal antibody EB-A2 labelled with peroxidise), and added to microtiter plates precoated with the anti-GM monoclonal antibody EB-A2. Plates were incubated for 90 min at 37 ºC, washed, and incubated with 200 µl of tetramethylbenzidine solution for an additional 30 min in the dark. Reactions were stopped with 100 µl of 1.5 M sulphuric acid, and optical densities read at 450 and 620 nm. Samples were run with positive, negative, and threshold controls supplied by the manufacturer. The optical density index was calculated as the optical density of the sample divided by the mean optical density of 2 threshold controls.

2.6.2 β-D-glucan (Fungitell® BG assay, Associates of Cape Cod)

The test was carried out according to the manufacturer's instructions. In brief, 5 µl of serum was transferred to the designated microplate wells and 20 µl of pretreatment reagent (an equal mixture of 0.25 M KOH and 1.2 M KCl) added and incubated for 10 min at 37 ºC. One hundred microlitres of Fungitell® reagent (eliminates Protein C which can also activates this pathway) was added to all wells containing controls and sera and shaken for 10 seconds. The plate was inserted into a microplate reader and read at 405/490 nm for 40 minutes at 37 ºC using Vmean kinetic analysis every 10 seconds (Gen5 2.0,

BioTek). Samples were run in duplicates and the concentration of BDG was calculated using calibration curves.

Sample selection

Not all samples were processed due to cost and time considerations. A selection of samples was taken to include (1) GM positive cases (n=131), (2) those with accepted CT signs but negative GM, i.e., possible IFD (n=41) (3) and negative controls (n=81) which were selected randomly from cases that had no evidence of IFD.

2.6.3 Lateral Flow Device (LFD)

The initial testing was based on the original work of Thornton [313] who supplied the LFD kits as part of our collaborative effort. The LFD did not contribute to the final EORTC status of study patients. A total of 988 serum samples from 52 patients were tested which included all proven and probable cases as well as some controls (no evidence of IFD). Fifty microlitres of serum samples were mixed with 50 µl buffer (tissue culture medium with 10% sodium azide) and the mixture applied to the LFD devices and results were recorded as negative, weakly positive, or strongly positive depending the strength of the reaction on the test line. A further modification was later adopted in an attempt to increase the contrast between the background and reaction line. In that modification 50 µl of serum was added to 100µl of PBS containing 4% (w/v) sodium EDTA and heated in a boiling water bath for three minutes and centrifuged for 5 minutes at 10,000 g. After this, 100 µl of the supernatant was added to the LFD and results recorded as above.

Sample selection

Sequential serum samples from recruitment to end of study period were collected from 29 proven/probable IFD, 6 possible IFD, and 21 not classified patients. All proven moulds were included except the cardiac case (case 7, Section 3.3) as no sera were available at the time of diagnosis.

2.6.4 PCR (Consensus)

The protocol was based on the work of White et al using bead beating extraction method on whole blood sample.[320] The Asp 2 primers targeting the 28S ribosomal DNA of *Aspergillus spp* and probes on the Rotorgene 6000 was used. The PCR results did not contribute the EORTC score.

Sample selection

Sequential whole blood (in EDTA) samples were tested from 52 patients with proven/probable IFD (16), possible IFD (4), not classified (24), and no evidence (8). Serum samples were tested from 16 patients with proven/probable IFD (10), not classified (3), and no evidence (3). Of these 16 patients 13 also had blood PCR.

2.6.5 Cytokine profiling (Luminex 30-plex panel, Invitrogen®)

This was done at baseline and every two weeks during inpatient stay for all study patients. The multiplex bead solid phase sandwich immunoassay method was used to measure 30 cytokines, chemokines and growth factors on the Luminex® 100™ machine on a 96 well plate capable of analyzing 80 samples in each run. The 30-Plex assay included: G-CSF, GM-CSF, HGF,

INF-α, IFN-gamma ,IL-1RA, IL-1β, IL-2, IL-2R, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12p40/p70, 1L-13, IL-15, ,IL-17, IP-10, MCP-1, MIG, MIP-1α, MIP-1β, RANTES, TNF-α and VEGF. The assay standards were prepared according to the manufacturer's instructions by combining 3 different premixed sets of protein standards reconstituted in 0.33 ml assay diluent. The standard curve was made by serially diluting reconstituted standards in 300 µL assay diluent to make 1:3, 1:9, 1:27, 1:81, 1:243, 1:729 dilutions and a blank. After pre wetting the plate and drying using the vacuum manifold, 25 µL of the 30-Plex beads of defined spectral properties, conjugated to analyte specific antibodies, were added to the wells. The beads were soaked in 0.2 ml Working Solution for 15-30 seconds and then washed twice. Fifty microlitres of incubation buffer was added into each well. To the wells designated for the standard curve, 100µL of the appropriate standard dilution was added. For the other wells designated for the samples, 50µL of Assay Diluent + 50 µL of sample were added and these were incubated for 2h at room temperature on an orbital shaker. During this incubation, analytes bind to the captured antibodies on the beads.

The Biotinylated Detector antibody (100µl) was added to all the wells and incubated for one hour at room temperature to allow the antibodies to recognize their epitopes and bind to the appropriate immobilized analytes. The excess biotinylated detector antibodies were removed and streptavidin conjugated to the fluorescent protein (Streptavidin-RPE) added and incubated for 30 minutes. During this incubation the streptavidin-RPE binds to the bionylated detector antibodies associated with the immune complexes on the

beads to form a four-part solid phase sandwich. After washing to remove the excess Streptavidin-RPE, the beads were analysed based on their spectral properties and the amount of associated fluorescence.

2.6.6 Genetic polymorphisms

Nine previously identified single-nucleotide polymorphisms (SNP) in TLR1 (rs5743611, 239C→G, T80R), TLR2 (rs5743708, A→G, Q753R), TLR3 (rs3775291, 234C→T, L412F), TLR4.1 (rs4986791, 1363C→T, T399I), TLR4.2 (rs4986790, 1063A→G, D299G), TLR6 (rs5743810, 745C→T, P249S), CLEC7A [dectin-1] (rs16910526, A→C, Tyr238X), CARD9 (rs4077515, 49499C→T, N12S) , and INF-γ (rs2069705, G→A, U/N) were analysed on samples from 189 patients (83 allogeneic HSCT recipients, 62 allogeneic donors, and 44 autologous HSCT recipients). Genotyping was carried out as described previously.[62,63,66,122,322-324]

2.7 Radiology methods

All scans were performed with multi-slice, thin-section, continuous volume acquisition technique and reconstructed with 5 mm slice thickness using high-spatial-frequency reconstruction computer algorithm. All scans were performed on the same scanner (Siemens Somatom Sensation 16). At recruitment into the study, all patients were required to have non−contrast enhanced thoracic CT study unless a previous scan was performed within two weeks for clinical reasons. Where a diagnostic scan was required due to failure to respond to second-line antibiotics, an initial low dose scan was performed (similar to

baseline scan) and if a focal lesion was found this was followed by contrast studies involving the affected area of the lungs. Patients with allergy to iodinated contrast or creatinine > 150µmmol/L were excluded. Intravenous iodinated contrast (70 ml Omnipaque™ at the rate of 4 ml/sec) was administered. One minute after the injection of contrast, the affected area of the lungs was scanned at 1 min, 2 min, 3 min, 5 min, and 10 min. The degree of contrast-enhancement was measured in Hounsfield units (HU). Three overlapping circular rings were placed on the nodule of interest and the HU recorded for each from pre-contrast and serially from 1 min to 10 min post contrast. The averages of these means were recorded. In addition, similar measurements were obtained from normal lungs for each phase of enhancement. The degree of contrast enhancement was calculated by subtracting the HU of normal lungs from the HU of the nodule for each phase.

Scoring

All the scans were reported by radiologists as part of the clinical services. In addition, abnormal baseline scans and all diagnostic and follow-up scans were reported and scored in a semi quantitative manner by study radiologists. Two consultant chest radiologists with more than 10 years' experience reviewed and scored the scans independently and where discrepancies existed, the final decisions were made by consensus. Two semi quantitative scoring systems were used for baseline/diagnostic scans and follow up scans.

Diagnostic scans

Each lobe was scored for EORTC accepted clinical signs (nodules, mass, halo and cavity) as well as non-EORTC signs (ground glass opacity, micronodule, bronchiectasis, bronchial wall thickening, and tree-in-bud) using the current Fleischner Society definitions.[325] Table 14 shows the layout of the proforma used for scoring. Each scan was independently scored by each radiologist and the average scores were compiled as the final score. If a particular score was more than 50% discordant, then the two radiologists agreed on a consensus score.

Table 14: Scoring proforma used for scoring diagnostic scans

	Nodule (0-2)	**Mass (0-2)**	**Halo (0,1)**	**Cavity (0,1)**	**GGO**	**consolidation**	**microdonule**	**BxExt**	**BxSev**	**BxWT**	**TiB**
RUL											
LUL											
RML											
Lingula											
RLL											
LLL											

Nodules or mass: 0= none, 1= ≤ 3, 2= ≥ 3; **halo or cavity**: 0= absent, 1= present; **GGO** (ground glass opacification): % of the lungs affected, **consolidation**: % of lungs affected; **micronodule**: % of the lungs affected; **BxExt** (bronchiectasis extent): 0= none, 1= 1 broncho-pulmonary (BP) segment, 2= > 1 BP segment, 3= generalized; **BxSev** (bronchiectasis severity): 0= none, 0.5= trivial, 1= 100-200 % of pulmonary artery (PA); **BxWT** (bronchial wall thickening): 0= none, 0.5= trivial, 1< 50% PA, 2= 50-100 % of PA; **TiB** (tree-in-bud): 0= none, 1= 1 or part of BP segment.

Follow-up scans

A similar approach was used in scoring the follow-up scans at about two weeks (follow-up 1), four weeks (follow-up 2), or longer (follow-up other). The scores were based on the EORTC/MSG response criteria.[321] Four possible outcomes were recorded: complete response (CR) [complete radiological resolution]; partial response (PR) [≥25% reduction in diameter of lesion(s); stable (0-25% reduction in diameter of lesion(s); progression (new lesions or worsening of pre-existing lesion(s). Each follow scan was scored individually by the two radiologists and the mean or consensus score (if the difference between the individual scores was >50%) was reached.

2.8 Biopsy

Lung biopsies were performed using the video-assisted thoracoscopic surgery (VATS) technique where possible. Open lung biopsy was reserved for cases where VATS was technically difficult or inappropriate. To increase the diagnostic accuracy of the biopsy, especially if the lesion was small, the procedure was preceded by CT-guided methylene blue 'tattooing' of the lesion as previously described.[284] The decision to use methylene blue was a joint decision between the cardiothoracic surgeon, radiologist and haematologist. Briefly, the patients were placed on the CT table in a position that allowed the shortest access to the lesion and the target nodule was identified by preliminary scans. Lignocaine (1%) was used for local anaesthesia. Using 20G needles, 0.8-2.0 ml of sterile 1 % methylene blue dye (Martindale, UK) with equal volume of iodinated contrast was injected into the vicinity of the

target lesion and along the track as the needle was removed, including the visceral pleural surface and the overlying chest wall. The diameter of targeted nodules, perpendicular distance from the pleural surface and complications of the procedure were recorded. The patients were then transferred to the operating theatre and the illustrative CT scans were provided for the surgeon. The VATS procedure was performed under general anaesthetic. The surgeon visualized the track as well as the methylene blue on the pleural surface. A wedge resection was performed and the specimens were sent for histology, microbiology and a separate fresh frozen sample for research (stored in the tissue bank at -80 ºC).

Between March 2009 and February 2011 65 potential biopsies were identified, 39 were performed and 38 were evaluable in 32 patients (one biopsy n=27; two biopsies n=4; three biopsies n=1) (Figure 9). There were 26 cases that were unfit for surgery due to poor clinical state of the patients (n=13), interference with chemotherapy/transplant schedule (9), and lack of theatre/surgical time (n=4). Slides were unavailable for review in one patient.

The majority of evaluable biopsies (82%) were performed using VATS procedure under general anaesthesia.[280] A wedge resection was performed and the specimens were sent for histology (fixed in formalin) and microbiological culture and sensitivity (not fixed in formalin). Five of the proven biopsy samples were sent to the Mycology Reference Laboratory in Bristol for Panfungal PCR and nuclear ribosomal repeat region sequencing.[326,327]

Open lung biopsy was employed in one patient due to the anatomical position of the lesion and one of the VATS biopsies had to be converted to open biopsy due to intra-operative bleeding. The four extrapulmonary biopsies were performed on the hard palate (2), mediastinal mass by mediastinoscopy (1), and heart (1).

The biopsy results were reviewed by two histopathologists. A semi-quantitative reporting system was used: (1) primary and secondary histology patterns, (2) presence or absence of fibrosis, (3) infectious organisms seen or not, (4) presence or absence of any malignancy, (5) diagnosis, and (6) suggested aetiology. In the event of significant discrepancy a consensus was reached.

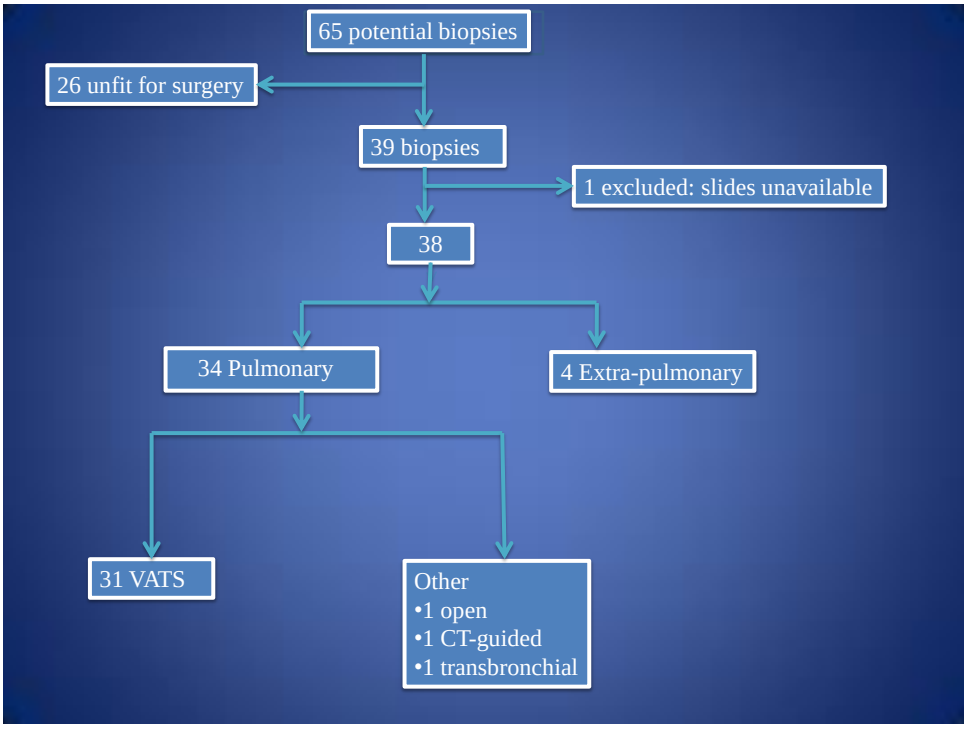


Figure 9: Outline of biopsy cases

2.9 Bronchoscopy

Transplant patients (both allogeneic and autologous) were offered baseline bronchoalveolar lavage before the initiation of the conditioning chemotherapy. The samples collected were sent for routine microbiology, virology, and biochemistry tests as well as galactomannan, β-D-glucan, and PCR. A uniform BAL protocol was used for all patients:

- 20ml bronchial wash with warm normal saline into bronchus intermedius followed by
- 150ml BAL into middle lobe in 50 ml aliquots
- Samples to Microbiology for MC+S, PCR, BDG, GM.
- Samples for cytokine profiling (Luminex) transported immediately on ice; centrifuged and the supernatant were stored at -80^{0}C and cell pellet re-suspended to analyse cellular components.

3.0 Database and Statistical analysis

All the patients recruited into the study had the following baseline details entered into our purpose-built database (IPAS): demographic details, reason for admission, past medical history including previous treatment details, disease status at admission, smoking and alcohol history, antifungal prophylaxis (type, dose, duration, reason for starting and stopping), antibiotics (type, dose, duration, reason for starting and stopping), CT scan results, bronchoscopy (where applicable), and clinical assessment (See CD for the full

database fields). Laboratory investigations included: FBC, U&Es, LFTs, Mg, CRP, galactomannan, β-D-glucan, PCR. Follow-up entry for the above tests was carried out on every patient as well as any changes in antifungal agents or antibiotics and the reason for such. Diagnostic and follow-up CT scan reports and biopsy results where available were also recorded. All patients were scored for evidence of IFD based on the EORTC criteria.

At the end of the study data was extracted from the database into ACCESS and exported into statistical software such as SPSS version 19.0 or Stata® version 11.2 for further statistical analysis. The incidence of IFD and IA was calculated for the whole cohort and for specific treatment categories, such as allogeneic HSCT, chemotherapy only, etc. Because each patient could have more than one treatment episode during the study, these incidences were based on treatment episodes rather than individual patients. Proven and probable IA were considered *bone fide* cases while possible IA was considered as a separate category. Cumulative incidences of IA and IFD were calculated at 100 days, six months and one year post index treatment. The index treatment was defined as the immunosuppressive/myelosuppressive therapy that the patient received prior to developing fungal infection. In case of no evidence of IFD the index treatment was the primary immunosuppressive/myelosuppressive therapy. The date of diagnosis was defined as the date in which the EORTC criteria were met and in the biopsy proven cases it was the day of the biopsy.

Risk factor profiles

Risk factors for IFD and death were examined using the Cox regression model.[328] Time to development of IA/IFD or death was used as the endpoint and censoring was the time of death or at the end of follow-up to obtain hazard ratios and 95% confidence intervals (CI). Independent variables tested in the univariate model included age, sex, performance status, ethnicity, occupation, smoking history, alcohol history, diagnosis, treatment received (chemotherapy, allogeneic HSCT, autologous HSCT, IST) disease status at initiation of therapy, number of prior therapies received, antifungal prophylaxis and treatment, duration of cytopenia (neutropenia, lymphopenia, monocytopenia), baseline CT findings. All variables examined in the univariate model with P values <0.10 were entered in the multivariate model and eliminated in a forward stepwise (Likelihood ratio) fashion. Adjusted Hazard ratios and 95% CI were calculated. Statistical significance was defined by two-tailed $P < 0.05$. The Kaplan-Meir method was used to estimate the overall survival (OS).[329]

Utility of diagnostic tests

For the purpose of this study the EORTC/MSG definitions were considered as the 'gold standard' for diagnosing IA with tissue diagnosis being considered the reference diagnostic test. Because of the difficulty of getting tissue diagnosis in the majority of patients the true disease status of the majority of patients remained unknown. To calculate the sensitivity, specificity and predictive values of the non-invasive tests used in this study using 2 x 2 tables, the following definitions were used:

1. Proven and probable IA were considered true positives

2. Proven non-IA fungal infections and those with no evidence of IA were considered true negatives

Cases of possible IA and those not classified under the current EORTC/MSG criteria were excluded.

Cytokines

The cytokine data was divided into baseline and follow-up 1 to 15 (sampling done at two weekly intervals during inpatient admission). Patients who were febrile at the time of initial samples or had already received chemotherapy were excluded. Stepwise logistic regression analysis was used for comparing the cytokine levels of those who eventually developed IA to those who did not. The mixed models [330] approach was used for assessing the diagnostic and prognostic value of repeated follow-up cytokine levels using binary outcome of proven/probable and no evidence of IA. The model was adjusted for clustering, age, sex, underlying diagnosis, treatment (allograft or autograft or chemotherapy), GVHD and bacterial or viral infection.

Radiology

The role of baseline CT scan (prior to starting immunosuppressive therapy) in predicting IPA was evaluated as above using the Cox proportional hazard model. The inter-observer agreement between the two radiologists in assessing diagnostic and follow-up scans was assessed by Cohen's Kappa coefficient [331] for categorical variables and the Bland-Altman plots [332] for

continuous variables such as the percentage of the lungs affected by consolidation or ground glass opacification. The value of contrast enhancement in the diagnosis of IPA was assessed by comparing the degree of enhancement in Hounsfield units between patients with proven/probable IPA and others with alternative diagnosis or not classified. Possible IPA was excluded from this analysis due to risk of misclassification.

Chapter 3 Results

3 Results

3.1 Recruitment

Recruitment started in December 2008 after ethical and R&D approval in October 2008. It started slowly partly to ensure all the recruitment process, sample collection, processing, testing and laboratory storage facilities were in place. By April 2009 recruitment peaked when 85% of all eligible patients were recruited (Figure 10).

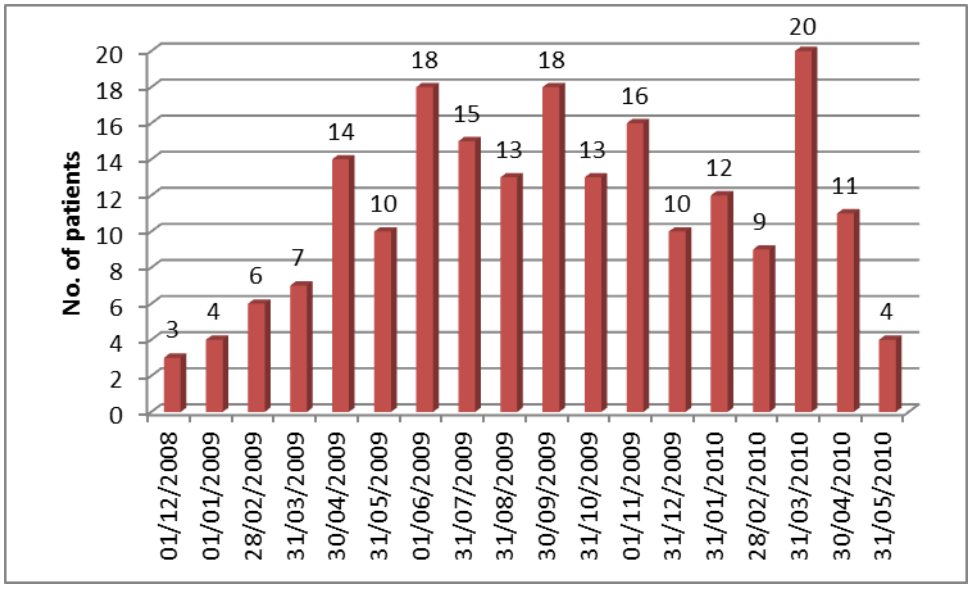


Figure 10: Recruitment

3.2 Patient characteristics

During the study period 203 patients with haematological malignancies undergoing allogeneic HSCT (n=99), autologous HSCT (n=66) or chemotherapy/IST (N=38) were recruited into the study. Their demographics

and clinical characteristics are reported in Table 15 according to the underlying treatment received. The total number of neutropenic febrile episodes was 409 with a median of 2 episodes/patient which was significantly more in the allogeneic HSCT and chemotherapy/IST patients compared to autologous HSCT patients (P<0.001). The median age was 54 years (range 19-73 years) and 123 (61%) were males. The ethnic origin was white (174; 86%), black (15; 7%), Asian (9; 4%), Chinese (2; 1%), and others (3; 2%). The median weight was 75 kg (range 47-134 kg). In total 95 (47%) were smokers of which 25 (12%) were current smokers while 149 consumed alcohol at a median of 5 units/week (range 2-70 units/week).

At admission into the study the median Karnofsky score was 90 (range 30-90) and median PAM score among the transplant candidates was 22. The CMV status was sero-positive in 89 (44%), negative in 96 (47%), and unknown in 18 (9%). The underlying malignancy was myeloid (n=111; 55%), lymphoid (46; 23%), and multiple myeloma (46; 23%). The disease status at admission was CR (56; 27%), partial remission (PR) [52; 26%], stable disease (42; 21%), progression/relapse (22; 11%), and new diagnosis (31; 15%). Prior to entering the study 172 (85%) received chemotherapy, IST or HSCT. In total 384 treatments were administered from current diagnosis (new diagnosis or relapse) and during the study period: allografts 106 (1 allograft n=93, 2 allografts n=5, 3 allografts n=1), chemotherapy (177), autografts (72), and IST (29). Two hundred and sixty-three (68%) of the treatments were given during the study period: allografts (106), chemotherapy (77; 1 cycle n=20, 2 cycles n=10, 3 cycles n=4, 4 cycles n=2, 5 cycles n=1), autografts (67), IST (13).

Among the 106 allografts the donor source was from siblings (25; 24%), volunteer unrelated donors (73; 69%), cord blood (5; 5%), haplo-identical (2; 2%). One patient died before stem cell infusion. GVHD was seen in 37 (37%) patients, 28 (28%) of which were acute (grade I n=4, grade II n=15, grade III n=9) and 17 (17%) were chronic (limited n=10, and extensive n=7). The affected sites of the aGVHD were skin only (21), skin and gut (3), skin, gut and mouth (2), skin, gut and eyes (1) and gut only (1). The sites of cGVHD were skin (11), skin and gut (2), skin, mouth, gut (1), gut (2), and liver (1). The median time for the development of aGVHD was 62 days (range 19-104 days) and for cGVHD 159 days (range 117-224 days) from day of stem cell infusion.

Of the 37 patients who developed GVHD 26 (70%) were treated with prednisolone at a starting dose of 1-2mg/kg/day and were treated for a median of 37 days (IQR 17-93 days). Additional immunosuppressive therapy was added in 3 of these patients: CSA (1), CSA, mycophenolate mofetil (MMF) and photopheresis (1), and infliximab (1). Thirteen of the 14 ITU admissions died; 12 in ITU and one after discharge. Their EORTC/MSG status was *A. fumigatus* (1), candida spp. (2), probable IFD (2), possible IFD (2), and not classified (7). Significant renal impairment (creatinine ≥ 240μmol/L) at the time of EORTC/MSG classification was seen in 4 patients while hepatic impairment was seen 35 patients.

Table 15: Demographics and clinical characteristics of the 3 patient groups

Characteristic	Allogeneic (N= 99)	Autologous (N=66)	Chemotherapy/IST (N=38)	P value
Age, mean (95% CI), y	48 (46-51)	58 (55-60)	48 (44-53)	<0.001
Male, n (%)	61 (62)	39 (59)	23 (61)	0.934
Diagnosis, n (%)				0.920
MDS/AML	65 (66)	0 (0)	19 (50)	
NHL	6 (6)	17 (26)	6 (16)	
Myeloma	2 (2)	44 (68)	0 (0)	
AA	13 (13)	0 (0)	6 (16)	
ALL	4 (4)	0 (0)	3 (8)	
HD	1 (1)	5 (8)	2 (5)	
Others	8 (8)	0 (0)	2 (5)	
EORTC/MSG classification*				<0.001
Proven mould	7 (7)	0 (0)	2 (5)	
Proven candida	5 (5)	0 (0)	0 (0)	
Probable	17 (16)	3 (5)	10 (26)	
Possible	13 (12)	6 (9)	7 (18)	
Not classified	40 (38)	24 (36)	12 (32)	
No evidence	23 (22)	33 (50)	7 (19)	
Concomitant infections, n(%)				
Bacteria	9 (9)	5 (8)	4 (11)	0.886
Viral	24 (24)	0 (0)	0 (0)	<0.001
Alive, n (%)	58 (59)	61 (92)	20 (53)	<0.001
Febrile episodes (total 409)	259	75	75	-
Median (95% CI), days	2.5 (2.1-3.0)	1.1 (1.1-1.3)	2.0 (1.5-2.5)	<0.001
Febrile episode duration, mean(95% CI), days	28 (22-34)	12 (10-13)	22 (28-58)	<0.001
Receiving steroids, n (%)	48 (49)	4 (6)	13 (34)	<0.001
Steroid dose, mean (95% CI)				
Total dose, mg	772 (494-1049)	113 (0-244)	539 (177-901)	0.001
Duration, days	46 (31-62)	19 (8-30)	35 (18-53)	0.467
ITU admission, n (%)	11 (11)	0 (0)	3 (8)	0.030

*N=105 as 7 cases had 2 separate proven and probable IFD episodes and were counted twice and one patient was excluded as she had probable IPA at time of recruitment

The laboratory characteristics are shown in Table 16. Overall 93% of all patients developed neutropenia of <0.5 x10^9/l and 59.2% of patients had neutropenia greater than 10 days and this was not statistically significantly different between the 3 treatment groups (P=0.086). The mean CRP was significantly higher in the chemotherapy/IST group compared to the transplant patients (P<0.001; Figure 10). CRP correlated well with fever especially fever lasting ≥3 days (Figure 11).

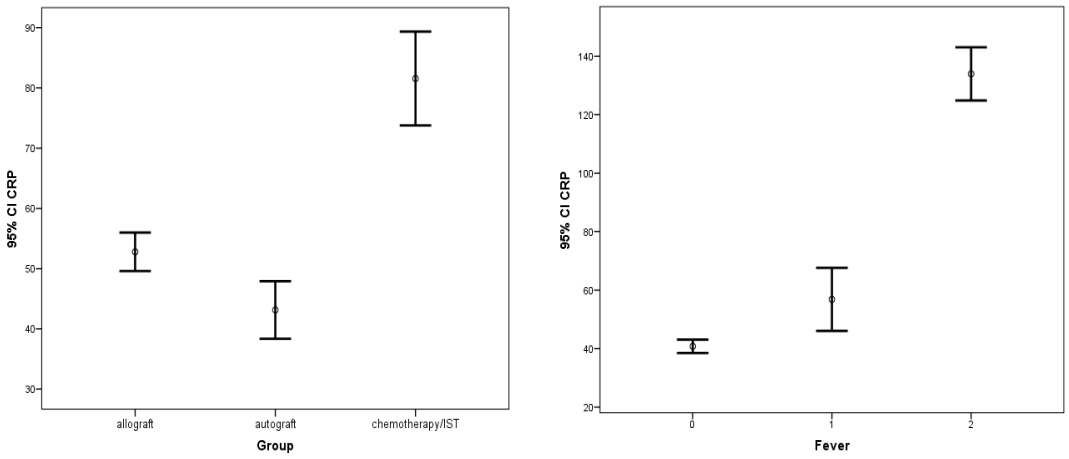


Figure 11: Error bar of CRP in the different treatment groups (left) and CRP with no fever (0), fever <3 days (1) and fever ≥ 3days (2).

Table 16: Laboratory characteristics of the 3 patient groups

Characteristic	Allogeneic	Autologous	Chemotherapy/IST	P value
Serum GMI (N=3086)				
Mean (95% CI)	0.2 (0.19-0.21)	0.19 (0.17-0.21)	0.32 (0.26-0.38)	<0.001
No.>0.5/no. tested (%)	98/2070 (4.7)	21/494 (4.3)	50/522 (9.6)	<0.001
Serum BDG (N=253)				
Mean (95% CI)	139 (120-159)	90 (63-117)	198 (153-243)	<0.001
No.>80/no. tested (%)	90/147 (61)	18/40 (45)	44/66 (67)	0.079
LFD (N=973)				
Total positive/no. tested (%)	32/745 (4.3)	0/42 (0)	3/186 (1.6)	0.094
Weak positive	11/745 (1.5)	0/42 (0)	3/186 (1.6)	-
Moderate positive	11/745 (1.5)	0/42 (0)	0/186 (0)	-
Strong positive	10/745 (1.3)	0/42 (0)	0/186 (0)	-
FBC, mean (95% CI)				
Haemoglobin (g/dl)	9.6 (9.4-9.8)	10.2 (10.0-10.4)	9.1 (8.9-9.3)	<0.001
Neutrophils ($x10^9$/l)	2.0 (1.8-2.3)	1.9 (1.7-2.1)	1.3 (0.9-1.6)	0.002
Platelets ($x10^9$/)	90 (77-102)	94 (80-109)	87 (63-111)	0.814
Lymphocytes ($x10^9$/l)	0.52 (0.41-0.63)	0.61 (0.54-0.69)	0.72 (0.57-0.87)	0.080
Monocytes ($x10^9$/l)	0.24 (0.20-0.27)	0.23 (0.21-0.26)	0.26 (0.18-0.34)	0.720
Leucopenia, N (%)				
Neutropenia anytime	88 (90)	64 (97)	35 (92)	0.227
Neutropenia >10d	64 (65)	32 (49)	24 (63)	0.086
Lymphopenia anytime	94 (96)	66 (100)	29 (76)	<0.001
Lymphopenia >10d	90 (92)	61 (92)	23 (61)	<0.001
Monocytopenia anytime	78 (80)	59 (89)	26 (68)	0.031
Monocytopenia >10d	43 (44)	9 (14)	16 (42)	<0.001
Duration of cytopenia, Mean (95% CI), days				
Neutropenia (N<0.5)	28 (19-38)	11 (9-14)	43 (28-58)	<0.001
Lymphopenia (<0.3)	74 (60-89)	20 (16-25)	39 (22-55)	<0.001
Monocytopenia (<0.01)	14 (9-19)	6 (5-7)	14 (8-20)	0.021
CRP, mean (95% CI) mg/l	53 (50-56)	43 (38-48)	82 (74-89)	<0.001

3.3 *Incidence of IFD*

During the study 45 proven and probable IFD cases were detected among 40 patients. One of the probable cases was excluded as she had probable IPA prior to entering the study, leaving 44 evaluable cases (Table 15). Seven patients had 2 separate episodes of IFD and were counted twice leading to total 209 cases at risk of IFD. The clinical characteristics of these cases and the other EORTC/MSG categories are shown in Table 17. Fourteen (32%) were proven and 30 (68%) were probable cases. *Aspergillus fumigatus* was seen in 2 cases and all the candidas were non-albican spp. (Figure 12). The median age of the IFD cases was 52 years, similar to non-IFD cases and 52% were male. Of the 44 IFD cases 29 received allogeneic transplantation (cord 5, sibling 8, 16 unrelated), 3 received autologous transplantation, and 12 received chemotherapy/IST.

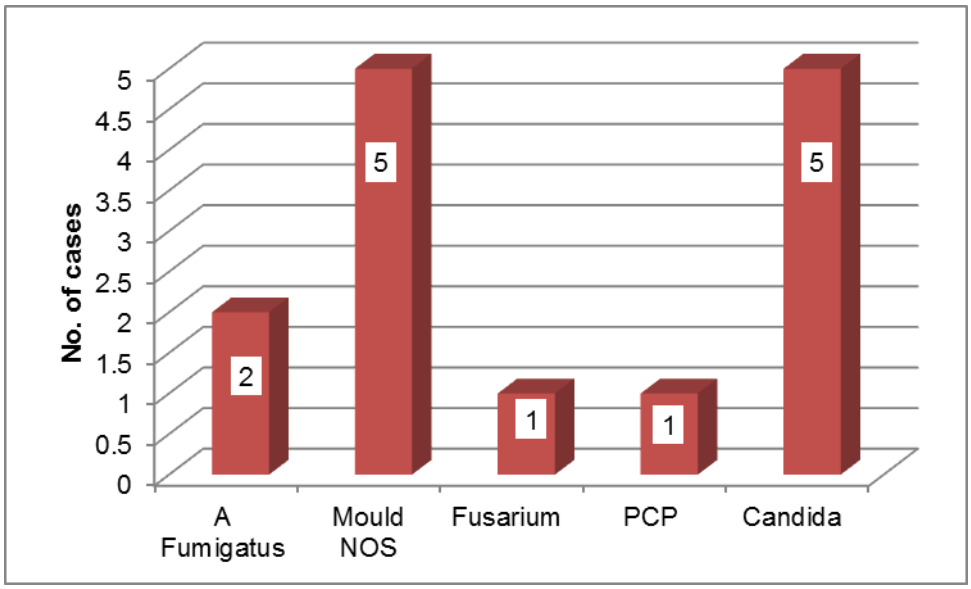


Figure 12: Proven IFDs

Table 17: Characteristics of the study cases according to the EORTC/MSG criteria

	Proven		**Probable**	**Possible**	**Not classified**		**No evidence**
Total N (%)	14 (7)		30 (14)	26 (12)	76 (37)		63 (30)
Subgroup	Mould	Candida	-	-	Serology	Radiology	-
No.of cases (%)	9 (4)	5 (3)	30 (14)	26 (12)	57 (27)	19 (10)	63 (30)
Mean age, (y)	52	52	50	48	51	54	51
Male Sex, n (%)	3 (33)	3 (60)	17 (57)	16 (62)	38 (67)	14 (74)	36 (57)
Diagnosis[§]							
MDS/AML	6 (67)	3 (60)	19 (63)	12 (46)	26 (46)	9 (47)	15 (24)
NHL	0 (0)	1 (20)	2 (7)	5 (19)	9 (16)	0 (0)	12 (19)
Myeloma	0 (0)	0 (0)	1 (3)	2 (8)	12 (21)	8 (42)	23 (37)
AA	0 (0)	1 (20)	2 (7)	3 (11)	5 (9)	1 (5)	7 (11)
ALL	1 (11)	0 (0)	3 (10)	1 (4)	2 (3)	0 (0)	0 (0)
HL	1 (11)	0 (0)	1 (3)	2 (8)	2 (3)	0 (0)	2 (3)
Others	1 (11)	0 (0)	2 (7)	1 (4)	1 (2)	1 (5)	4 (6)
Treatment*							
Allo	6 (67)	5 (100)	12 (40)	7 (27)	26 (46)	9 (47)	24 (38)
Auto	0 (0)	0 (0)	3 (10)	6 (23)	17 (30)	7 (37)	33 (52)
Chemo & IST	3 (33)	0 (0)	15 (50)	13 (50)	14 (24)	3 (16)	6 (10)

[§]P=0.003; *P<0.001

Excluding the non-Aspergillus-type cases the cumulative incidence of IA (proven and probable) was 37/209 (17.7%). IA cases were predominantly

myeloid, had more febrile neutropenic episodes and longer duration of neutropenia compared to non-IA cases (Table 18).

Table 18: Comparison of the clinical characteristics between IA and no IA patients

	IA (N=37)	**No IA (N=66)**	**P value**
Age, mean, years	51.1	51.7	0.762
Male, n (%)	20 (54)	38 (58)	0.730
Diagnosis, n (%)			<0.001
MDS/AML	23 (62.2)	17 (25.8)	
NHL	2 (5.4)	13 (19.7)	
Myeloma	1 (2.7)	23 (34.8)	
AA	3 (8.1)	7 (10.6)	
ALL	3 (8.1)	0 (0)	
HD	2 (5.4)	2 (3.0)	
Others	3 (8.1)	4 (6.1)	
No. of FNE/patient	3.2	1.2	<0.001
FNE, mean, days	44.3	11.7	<0.001
FBC, mean			
Haemoglobin (g/dl)	9.0	10.0	0.001
Neutrophils ($x10^9$/l)	1.4	2.0	0.587
Platelets ($x10^9$/)	64	95	0.141
Lymphocytes ($x10^9$/l)	0.6	0.5	0.446
Monocytes ($x10^9$/l)	0.3	0.2	<0.001
Duration of cytopenia, Mean, days			
Neutropenia (N<0.5)	38.6	18.6	<0.001
Lymphopenia (<0.3)	34.5	57.1	<0.001
Monocytopenia (<0.01)	22.0	8.7	<0.001

FNE: febrile neutropenic episode

Proven IFD

The 14 proven IFD cases are shown in Table 19 and 4 patients were further discussed.

Table 19: Proven IFD cases

Case	Age(y)/ sex	Diagnosis	Treatment	Radiology	GM/ BDG	Histology/ BAL	Culture
1	26/M	MDS/FA	IST	Nodules/hard palate lesions	Pos/ pos	Mould IFD	Neg
2*	61/F	AML	Allo HSCT	Nodule	Pos/ pos	Mould IFD	Neg
3	28/F	MDS	Cord allo HSCT	Nodules+halo	Pos/ pos	Mould IFD	Neg
4	72/F	HL/AA	Chemo	Nodules+halo	Pos/ pos	Mould IFD	Neg
5	54/M	MDS	Allo HSCT	Nodules	Neg/ pos	Mould IFD	Neg
6	67/M	ALL	Chemo	Nodules+halo	Pos/ pos	Haemorrhagic foci	*Aspergillus fumigatus*
7	59/F	CLL	Allo HSCT	MRI: cardiac mass	NA	Mould IFD	*Aspergillus fumigatus*
8	54/F	MDS	Allo HSCT	Normal	Neg/ neg	-	Fusarium spp
9	43/F	MDS/FA	Allo HSCT	Nodules, cavity, GGO	Neg/ neg	*BAL: pneumocystis jiroveci*	Neg
10	59/F	AA	Allo HSCT	Nodules	Neg/ pos	-	*Candida parapsilosis*
11	69/M	AML	Allo HSCT	GGO	Neg/ pos	-	*Candida glabrata*
12	55/M	AML	Allo HSCT	Nodules	Neg/ neg	-	*Candida guilliermondii*
13	28/F	MDS	Allo HSCT	Nodules+halo	Pos/ pos	-	*Candida Kefyr*
14	47/M	NHL	Allo HSCT	Normal	Neg/ pos	-	*Candida guilliermondii*

*post mortem: disseminated mould. Tissue PCR failed to identify spp. NA; not available

Case 2- Disseminated invasive mould disease at post mortem

This 61 year old female with AML M0 with complex cytogenetics received reduced intensity conditioning (RIC) matched unrelated donor (MUD) allogeneic HSCT in May 2009. She achieved CR post allograft with full donor engraftment. However, her post-transplant period was complicated by grade II acute and then extensive chronic GVHD resulting in prolonged systemic immunosuppressive therapy, recurrent CMV reactivations requiring antiviral therapies, prolonged cytopenias and recurrent neutropenic sepsis, and pulmonary embolism six post post-allograft. She was diagnosed with probable IPA (GM and BDG positive) four months post allograft. Lung biopsy was planned but she became acutely unwell on the morning of surgery and therefore biopsy was not performed.

249 days post allograft she was admitted to her local hospital with diarrhoea, cough, progressive weight loss and deterioration in her renal and liver function tests. Two weeks after her admission she developed acute deterioration of her conscious level with Glasgow coma score (GCS) fluctuating between 7 and 10, bilateral ankle clonus, and upgoing planter on the right. She was then transferred to King's. She was on Posaconazole, Gancyclovir, Enoxaparin treatment dose, Dapsone, Cetirizine, Levothyroxine, Mycophenolate, Ranitidine, Azithromycin, Prednisolone, and Ciclosporin. Her total antifungal experience during the study period is shown in Figure 13.

Case 2: antifungal timeline

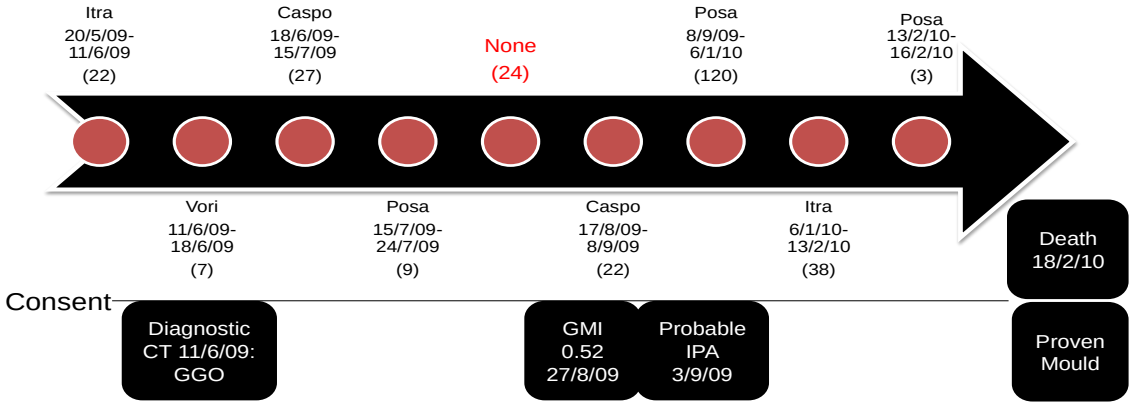


Figure 13: Antifungal experience of Case 2. She was on antifungals for a total of 248 days of her 274 patient days at a cost of £29,711.60.

Four days after admission in Kings she deteriorated further neurologically and became incontinent of urine and faeces, pupils became dilated and nonreactive bilaterally, and tone and reflexes were increased in all limbs. CT scan of her brain was performed and showed multiple bilateral low density lesions involving both grey and white matter (Figure 14). The differential diagnosis includes infection, neoplasia and multiple ischaemic lesions

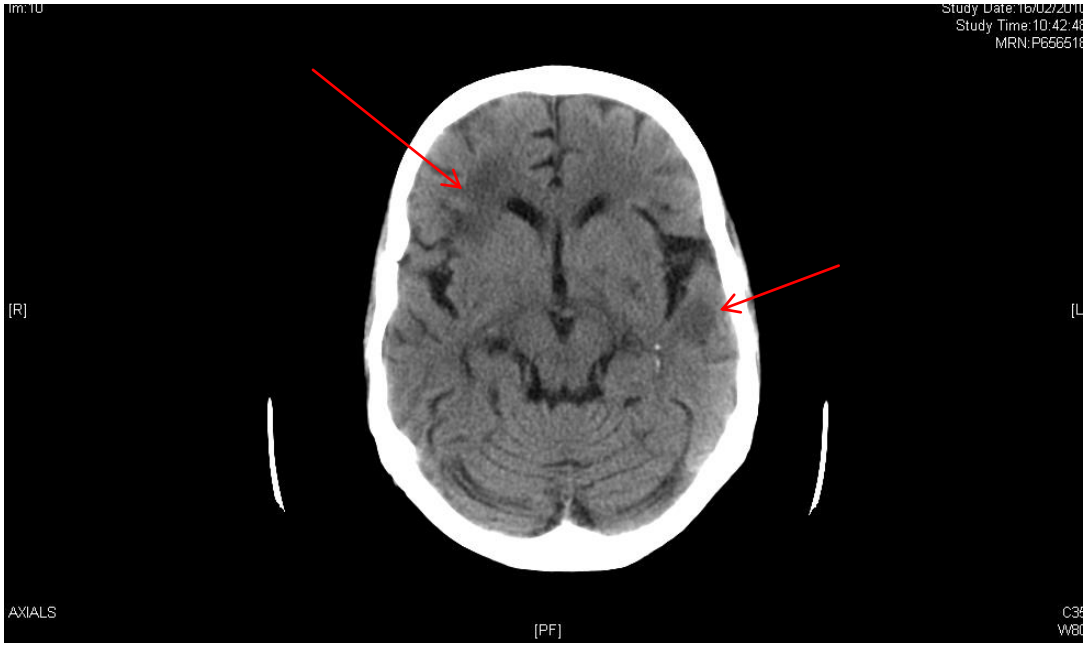


Figure 14: CT brain showing bilateral low density lesions in the right frontal lobe, predominantly involving white matter but extending to the insular, and anteriorly in the left temporal lobe, extending to the temporal operculum, the grey-white matter interface in the posterior right parietal lobe. No haemorrhage was identified.Unfortunately contrast could not be given due to the patient's poor renal function.

The patient died within 24h of the CT brain and a hospital postmortem was carried out. This showed:

1. Central nervous system

Multiple haemorrhagic infarcts of varying sizes involving the white matter and cortex were found (Figure 15). Theses lesions were present in the frontal and parietal lobes, insular cortex, basal ganglia and cerebellum.

Figure 15: Coronal sections of the brain showing haemorrhagic foci (courtesy of the neuropathology department, KCH)

Histology showed abundant fungal hyphae in the brain parenchyma and within blood vessels (Figure 16). The fungal hyphae appeared to be predominantly non-septated and branching predominantly at right angles. The fungi were infiltrating the walls of the blood vessels, and in places causing complete obstruction of vascular lumens (Figure 17). This affected both parenchymal and meningeal blood vessels; the latter was associated with neutrophilic infiltration of the meninges and fungal infiltration, consistent with fungal meningitis (Figure 18).

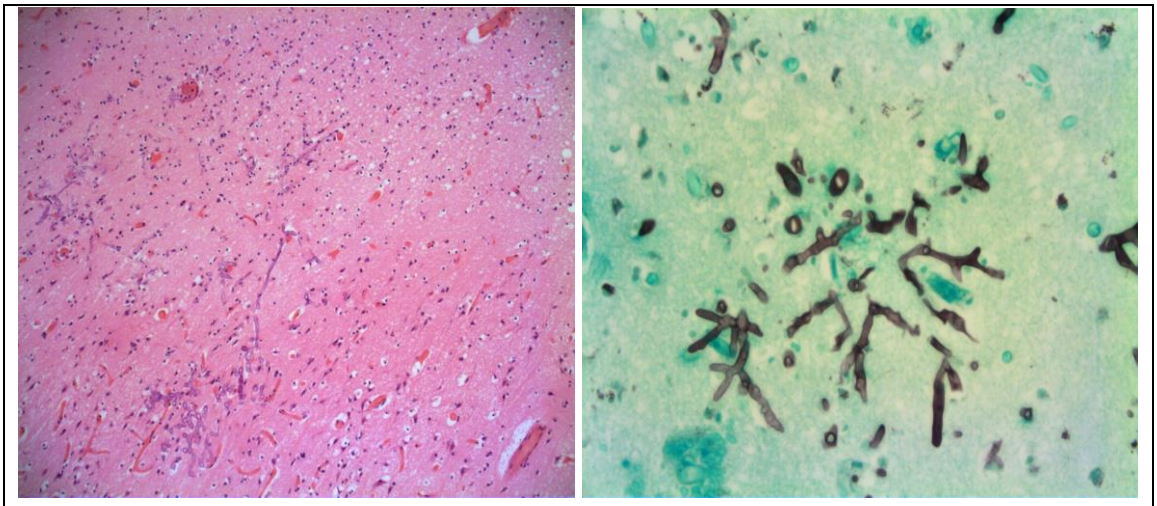


Figure 16: Right frontal lobe at PM stained with H&E (left) and Grocott (right) showing hyphal infiltration (courtesy of the neuropathology department, KCH)

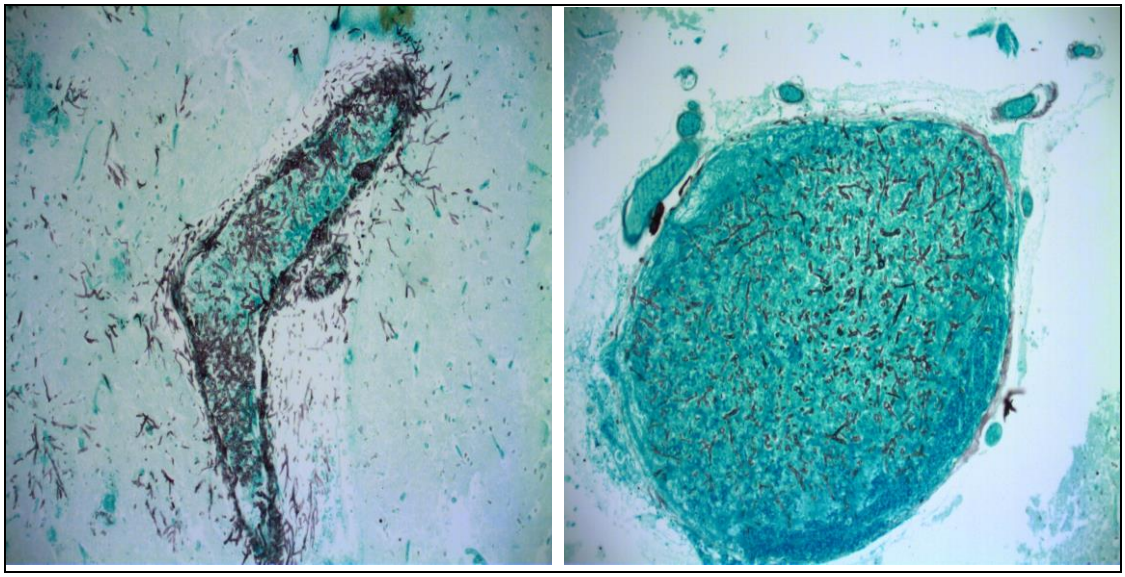


Figure 17: Gross angioinvasion of the brain (left) and occlusion of the cerebral artery (right) (courtesy of the neuropathology department, KCH)

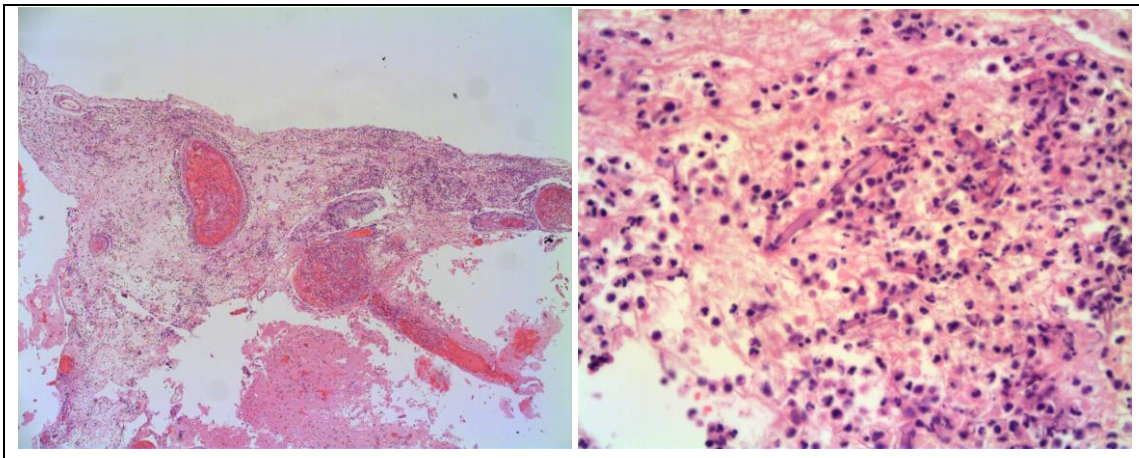


Figure 18: Fungal meningitis showing hyphael and neutrophilic infiltration of the meninges (courtesy of the neuropathology department, KCH)

2. Heart

Two soft to firm lesions with red peripheral boundaries and yellow central areas were seen in the posterior left ventricular wall and the anterior lateral left ventricle. The appearances were suggestive of a recent myocardial infarct. This would explain the chest pain that the patient experienced on her last admission. Histology confirmed cardiac invasive mould disease (Figure 19).

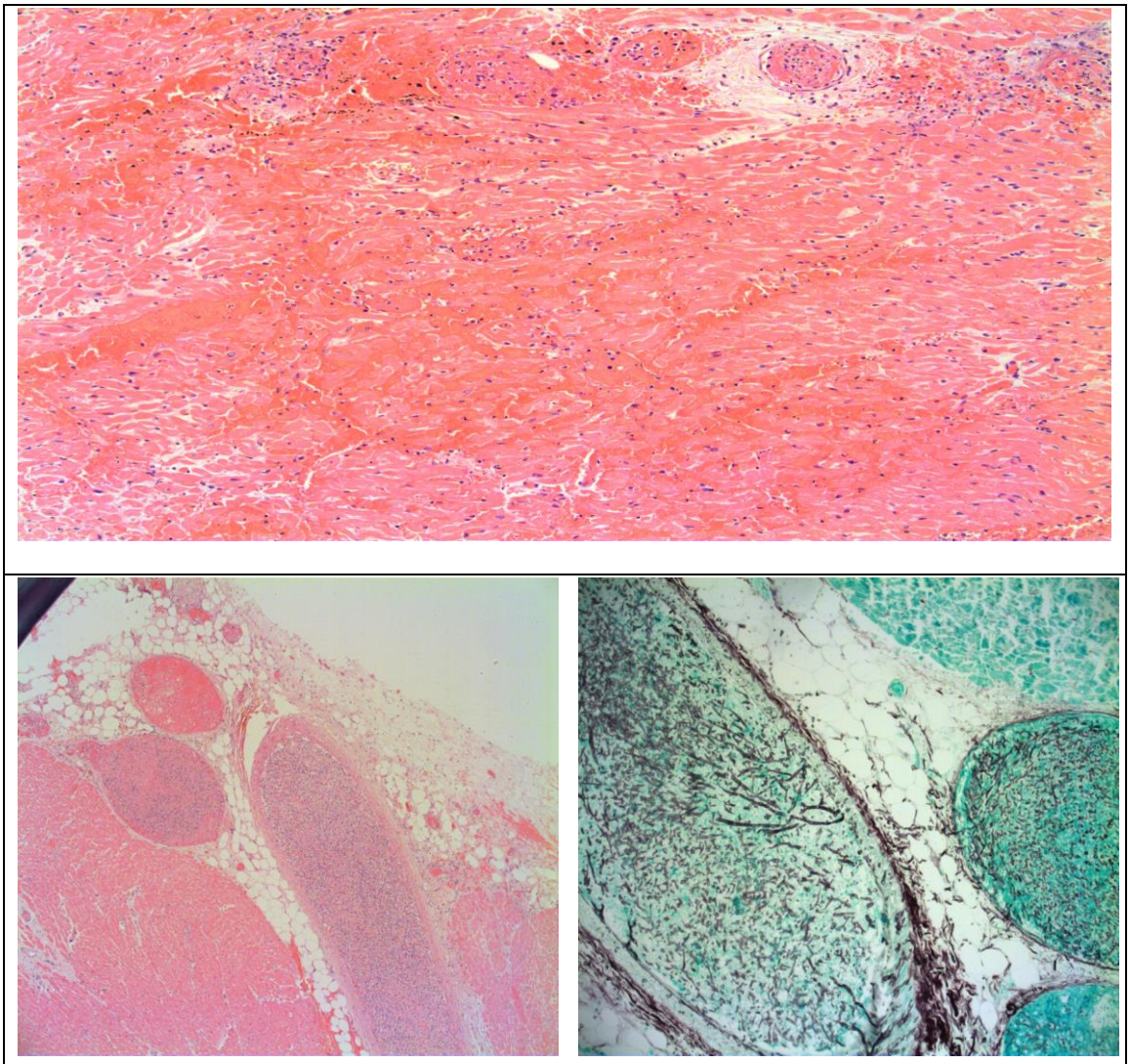


Figure 19: Post mortem findings on the heart. Top: low power view of the myocardium showing obstruction of the coronary arteries associated with foci of haemorrhagic changes, oedema, and neutrophilic infiltrate suggestive of acute myocardial infarction. Bottom: high power view of the coronary arteries stained with H&E (left) and grocott (right). Large luminal septic thrombi composed of fibrin and fungal hyphae are evident. (Courtesy of the neuropathology department, KCH)

3. Lungs

A triangular area of firm consistency was noted in the right upper lobe apex measuring 5cm in diameter with dark-red discoloration. This was associated with pulmonary emboli in the corresponding blood vessels and probably represented secondary organised infarcts. This was the likely cause of the PE

the patient was diagnosed with ante-mortem. Histology confirmed angioinvasive mould disease (Figures 20 & 21). Fungal cultures were negative.

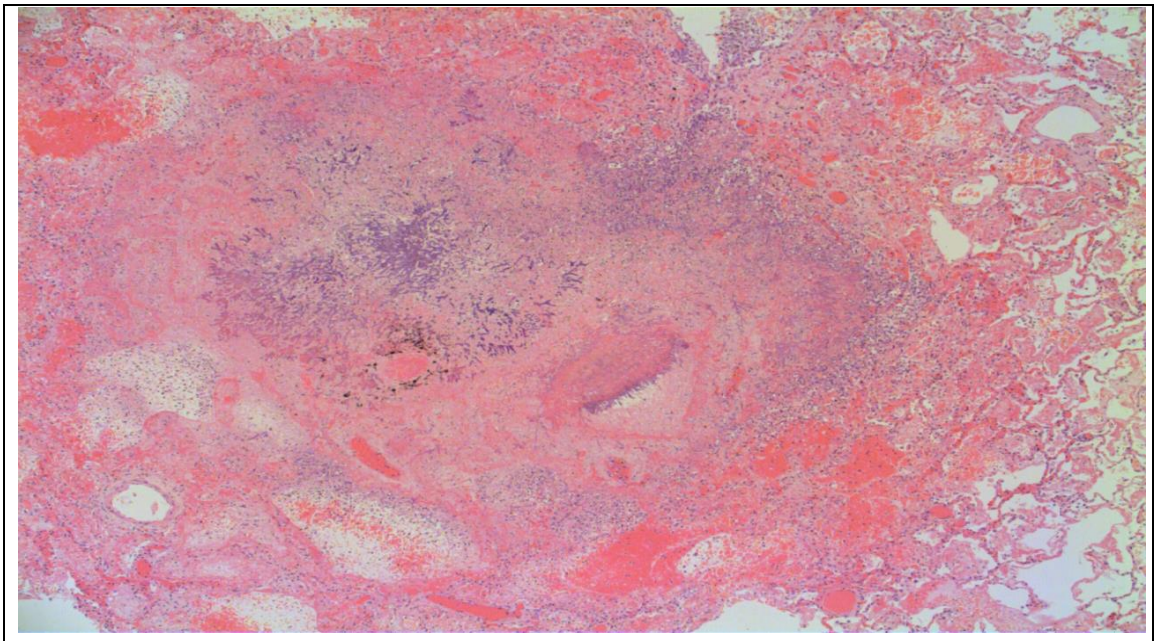


Figure 20: Coagulative necrosis of the lungs associated with numerous fungal hyphae (courtesy of the neuropathology department, KCH)

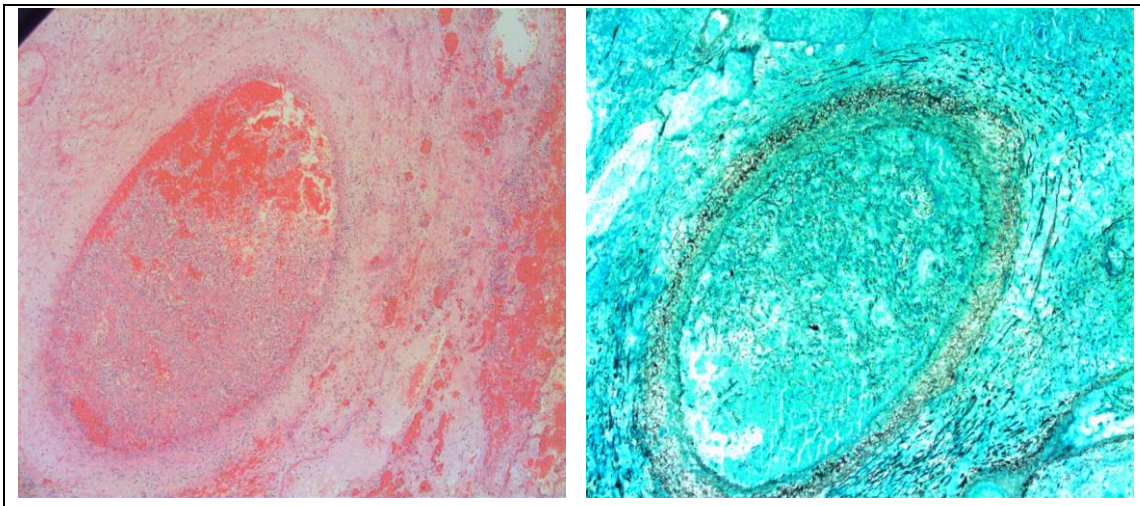


Figure 21: Septic emboli in the pulmonary vessels associated with ischaemic and haemorrhagic infarcts. Multiple small nodules of fungal balls are noted.

4. Liver

A mild degree of congestion and nutmeg appearance was noted. In addition, multiple pale/yellow irregular lesions were noted in parts of the liver. These were examined histologically and showed invasion by fungal hyphae (Figure 22).

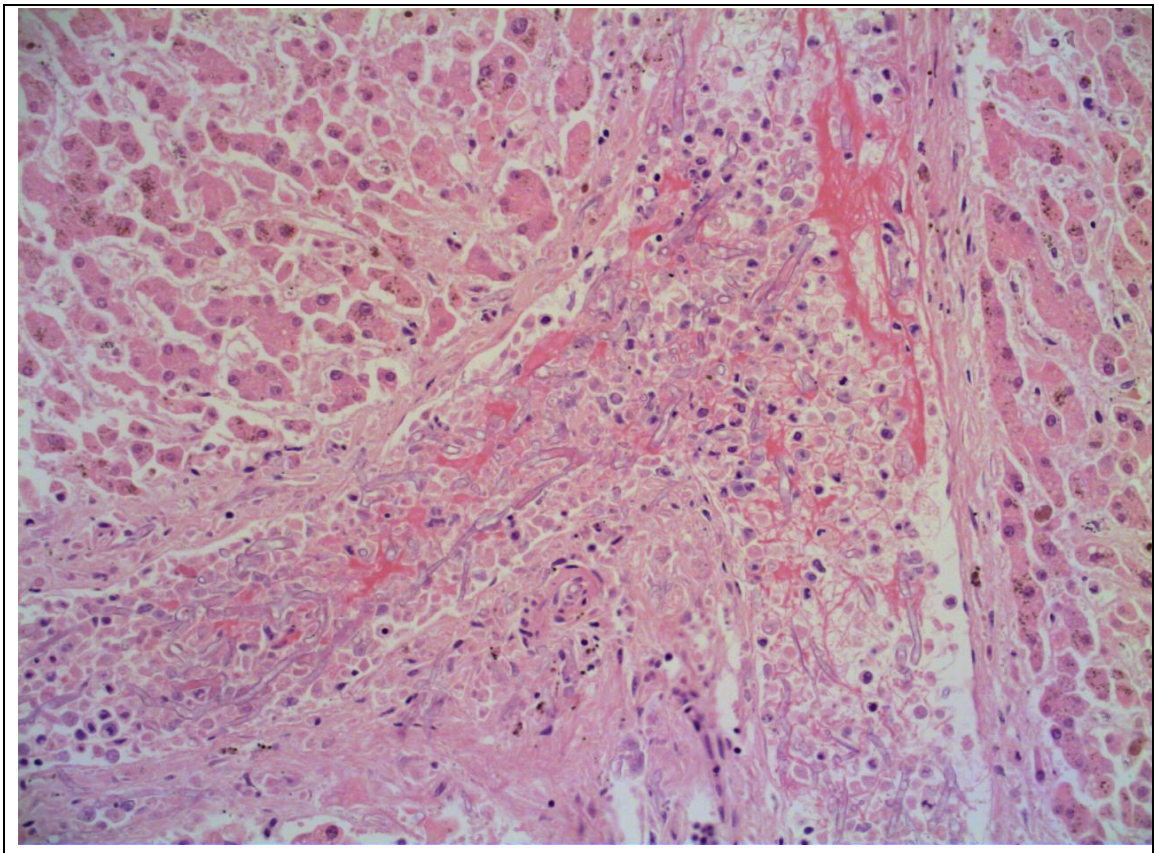


Figure 22: Hepatic invasive fungal disease. Congested blood vessels are noted around central veins associated with haemosiderin laden macrophages. Numerous branching hyphae are seen within the vascular lumen with surrounding area of infarction (courtesy of the neuropathology department, KCH).

5. Kidneys

There were a few haemorrhagic lesions on the cortices of the left kidney. A mild degree of thinning of the cortices was noted but there was no evidence of mass lesions. Histological examination confirmed renal involvement by invasive fungal disease (Figure 23).

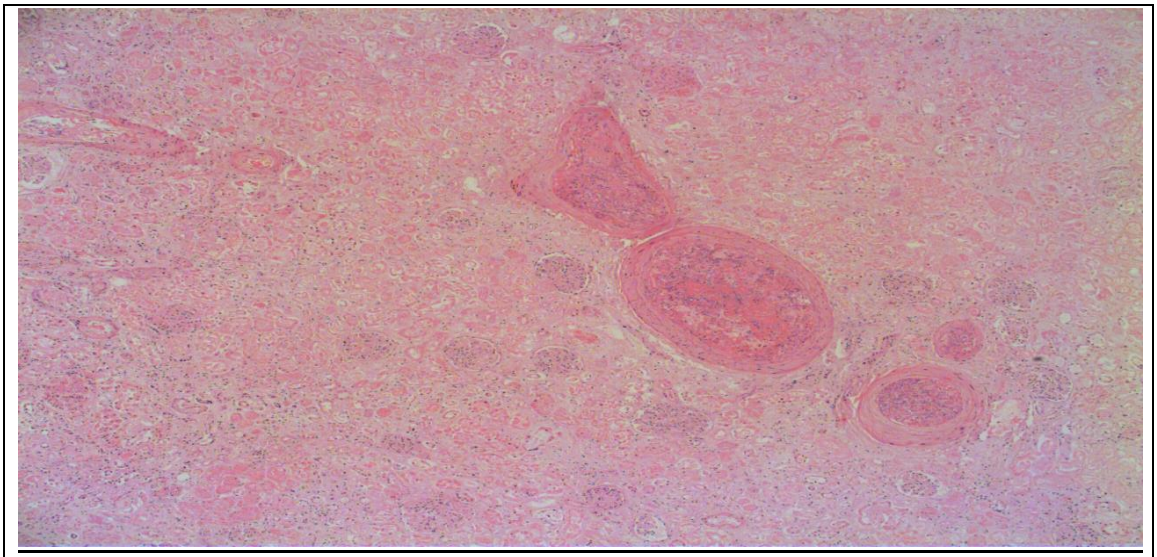


Figure 23: Intravascular fungal emboli associated with ischaemic renal cortical necrosis (courtesy of the neuropathology department, KCH)

6. Gastrointestinal tract

The stomach contained blood-stained material and showed multiple dark-red mucosa lesions of about 2cm in diameter. Similar lesions were noted in the small and large intestine. Histological examination confirmed GI involvement with the disseminated fungal disease (Figure 24). Fungal and bacterial cultures were all negative. Bone marrow examination showed hypocellularity but otherwise normal trilineage haemopoiesis with no evidence of relapse of AML. The cause of death was recorded as disseminated angioinvasive fungal disease.

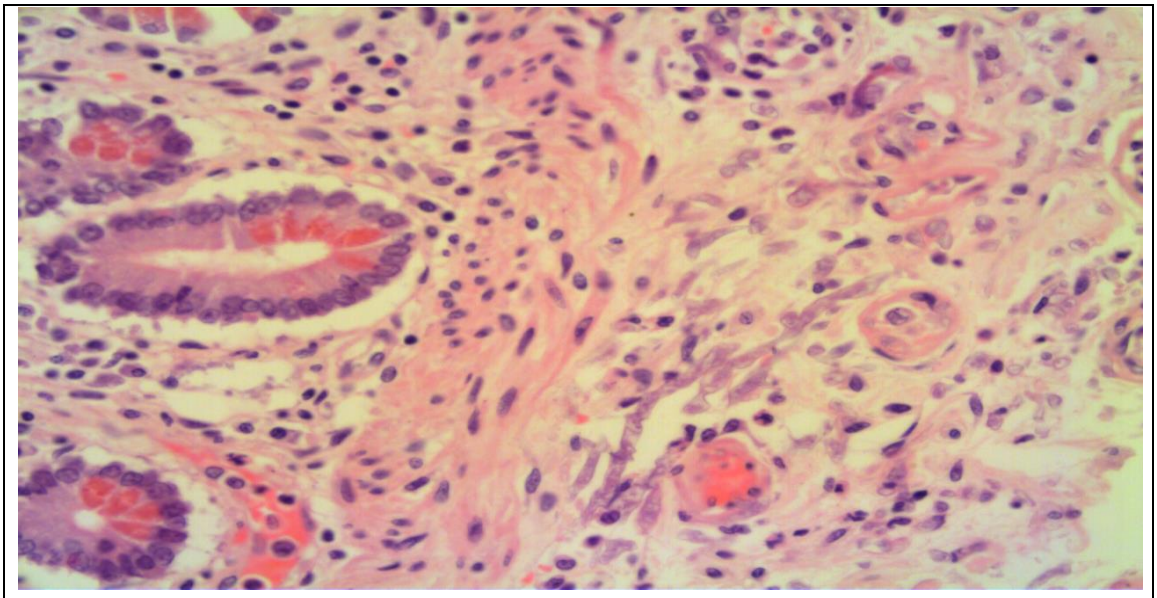


Figure 24: Duodenum showing infiltration by fungal hyphae (courtesy of the neuropathology department, KCH).

Case 4- Invasive pulmonary mould disease

This was a 72 year old female who was diagnosed with Hodgkin lymphoma in 2008 and treated with five courses of ChlVPP (chlorambucil, vinblastine, procarbazine and prednisolone) chemotherapy. This was followed by prolonged pancytopenia. She developed culture-negative neutropenic sepsis resistant to second line antibiotics. CT scan of the chest showed classic nodules with halo sign (Figure 25). Both GM and BDG were positive. Lung biopsy showed invasive mould disease (Figure 26) but fungal cultures were negative. She died from IFD.

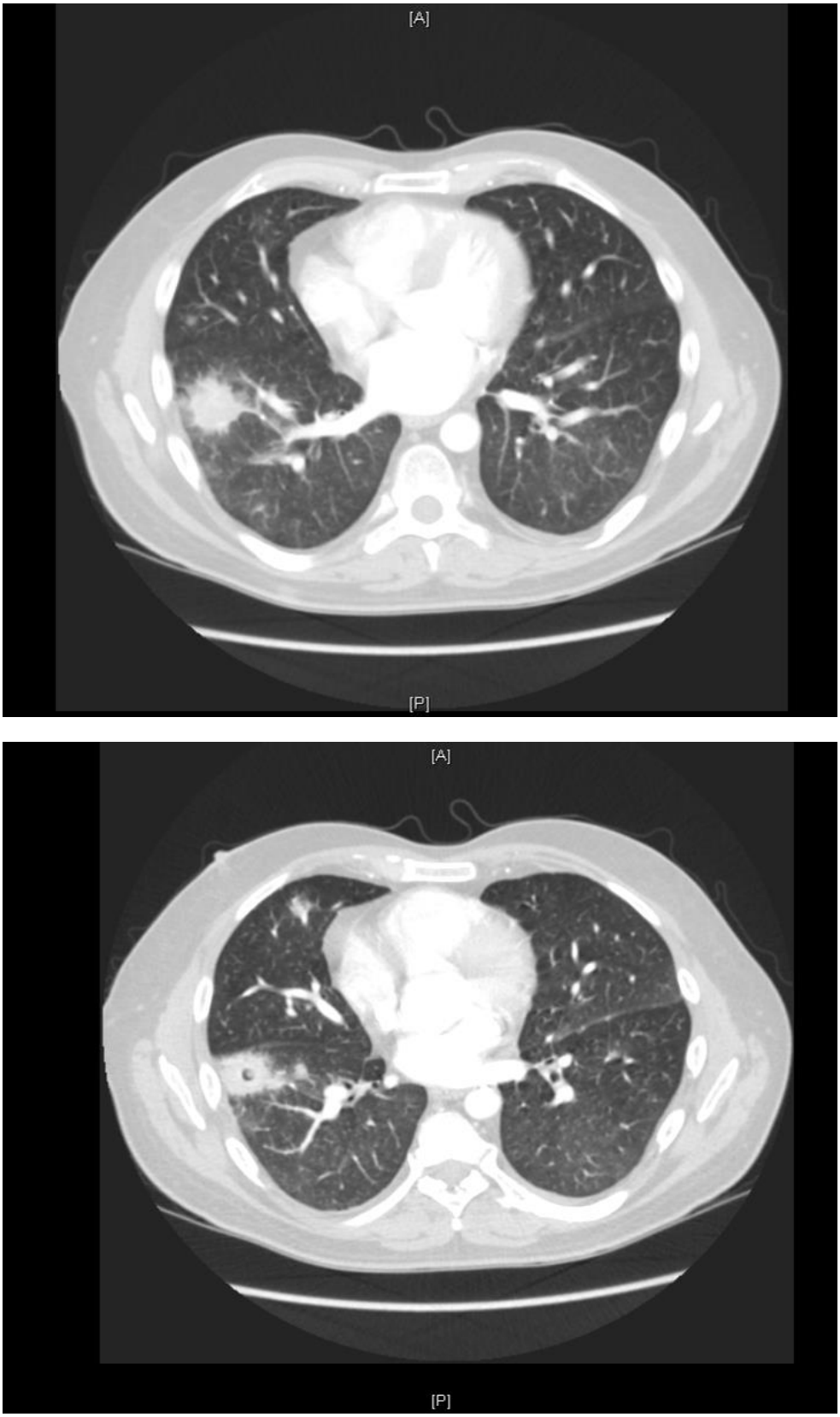


Figure 25: CT chest showing a macronodule with ‘halo’ sign (top) and air crescent sign (bottom)

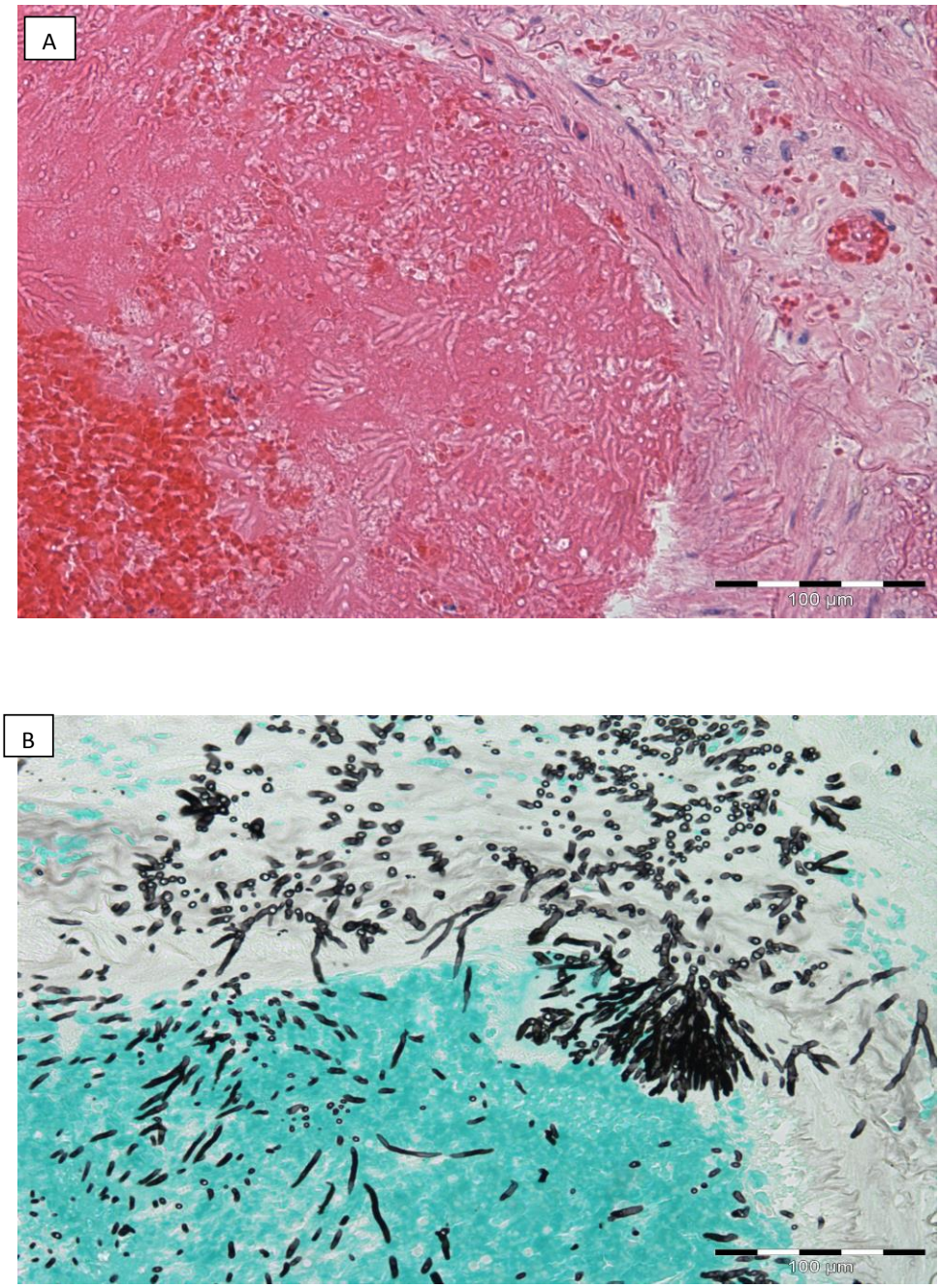


Figure 26: Angioinvasive pulmonary aspergillosis. The H&E shows extensive coagulative necrosis with angio invasion of the lung vasculature (A). The angio invasion is more obvious with silver staining which highlights numerous branching hyphae (B).

Case 7: Aspergillus endocarditis and angioinvasive cardiac disease

This was a 59 year-old female with P53-deleted chronic lymphocytic leukaemia (CLL) heavily pre-treated with chemotherapy including alemtuzumab. She underwent RIC MUD allogeneic HSCT using fludarabine/melphalan/alemtuzumab (FMC) conditioning in May 2010. This was complicated by gastrointestinal GVHD, CMV and adenovirus reactivations, bacterial infections (*Enterobacter cloacae* on bronchoalveolar lavage, recurrent *Pseudomonas aeroginosa* chest infections) and cytopenia. She had prolonged inpatient admission in her local hospital where she developed new cardiac murmurs during her many neutropenic sepsis episodes. Echocardiogram showed a subaortic mass with outflow obstruction. She underwent removal of this mass 258 days post allograft and the histology and culture confirmed *Aspergillus fumigatus* sensitive to amphotericin B, voriconazole, caspofungin and itraconazole (Figure 27). Despite combination therapy with Caspofungin and voriconazole she progressed and died of her sepsis.

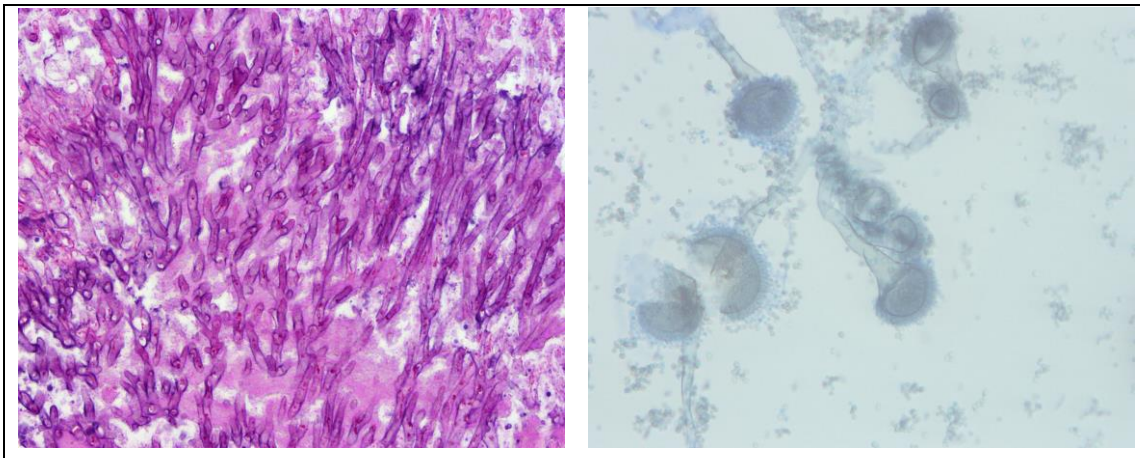


Figure 27: The branching hyphae of from histological examination of the heart (left) which was cultured and identified as *Aspergillus fumigatus* (right)

Case 8: Disseminated Fusariosis

54 year old female with high risk MDS underwent RIC MUD allogeneic HSCT in September 2009 using fludarabine/busulphan/alemtuzumab (FBC) conditioning. She had primary graft failure and underwent her second RIC allograft using an alternative donor and fludarbine/cyclophosphamide/alemtuxumb (FCC) conditioning in December 2009. She developed neutropenic sepsis and widespread skin lesions which typically started as violaceous lesions (Figure 28) before progressing to erythematous indurated lesions (Figure 29). *Fusarium* spp with typical morphological characteristics were repeatedly recovered from blood cultures (Figure 30).

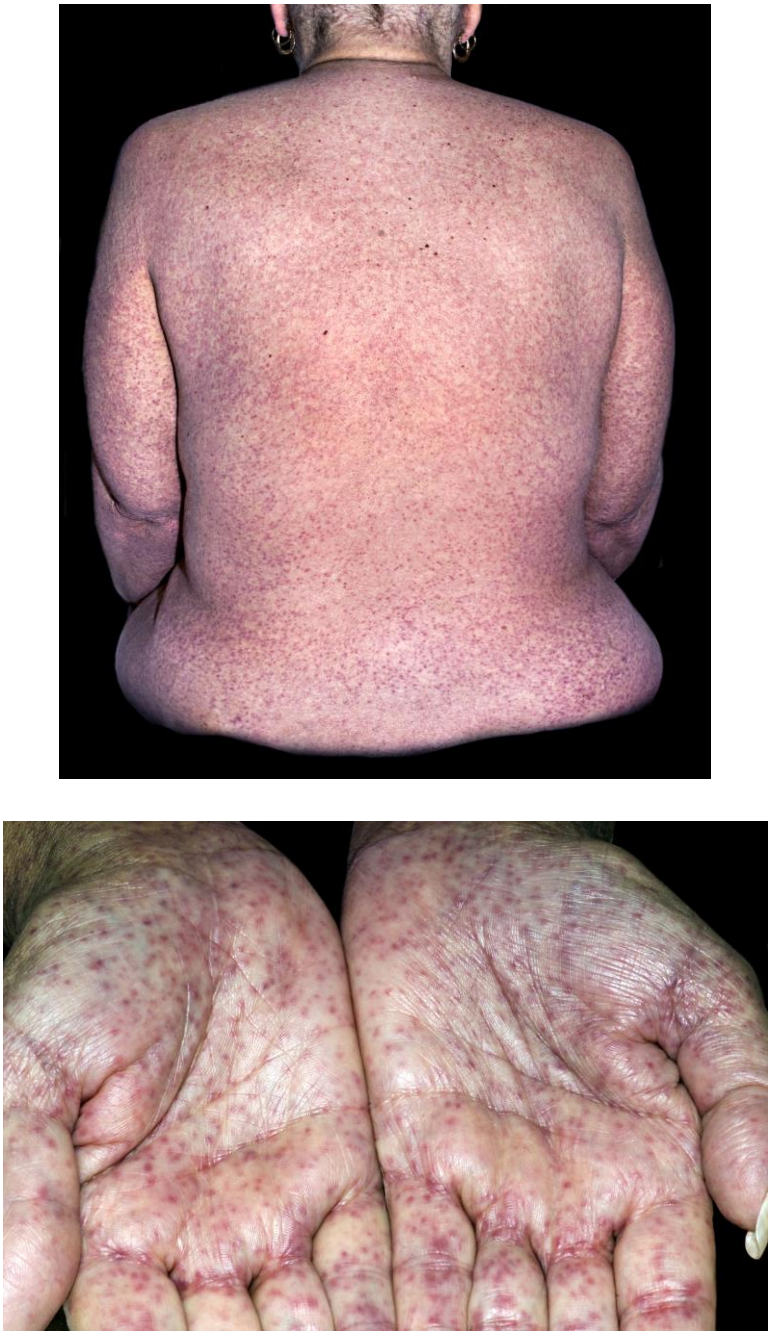


Figure 28: widespread violaceous skin lesions due to fusariosis. The lesions were particularly prominent on the trunk (top) and hands (bottom) (produced with patient's permission).

Figure 29: Erythematous indurated skin lesions with central necrosis (with patient's permission). These lesions were widespread but prominent on the upper limbs (top) and around the nose (bottom).

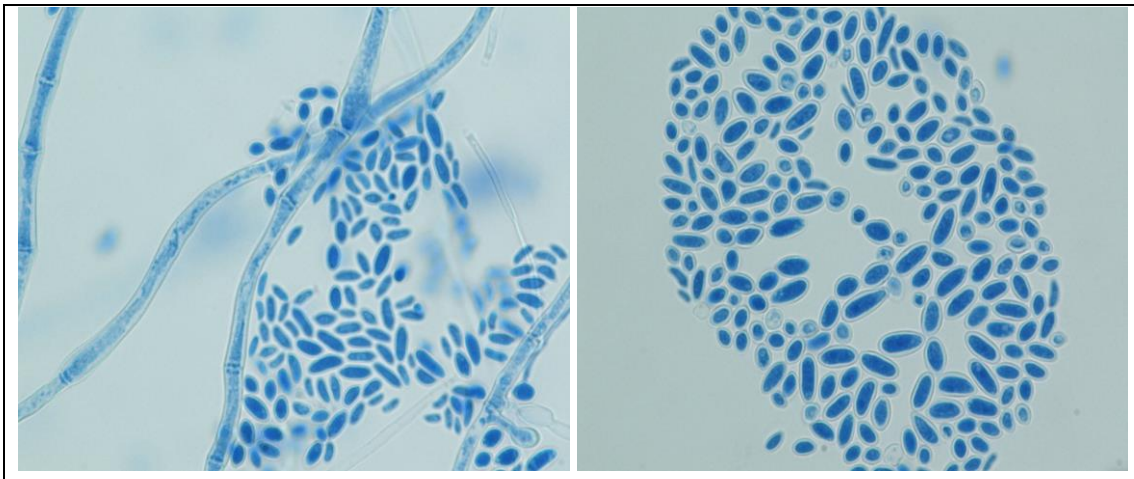


Figure 30: Blood cultures showing *Fusarium* spp with septate hyphae and conidia

3.3.1 Incidence of IFD depends on the diagnostic tools used

Using all available diagnostic tools recommended by the revised EORTC/MSG criteria, the study cases were classified into proven, probable and possible IFDs. However, a significant proportion of cases could not be classified as such (Figure 31). Patients who had normal CT scans and negative GM and BDG were considered to have no evidence of IFD (N=63). The 'not classified' group included patients who had positive GM and/or BDG but no associated radiological features (N= 57) or had radiological abnormalities other than those recognised by this classification (N= 19) [see also Table 17]. One of the proven mould cases (case 5, Table 19) would have been classified as possible IFD by GM alone without histological proof.

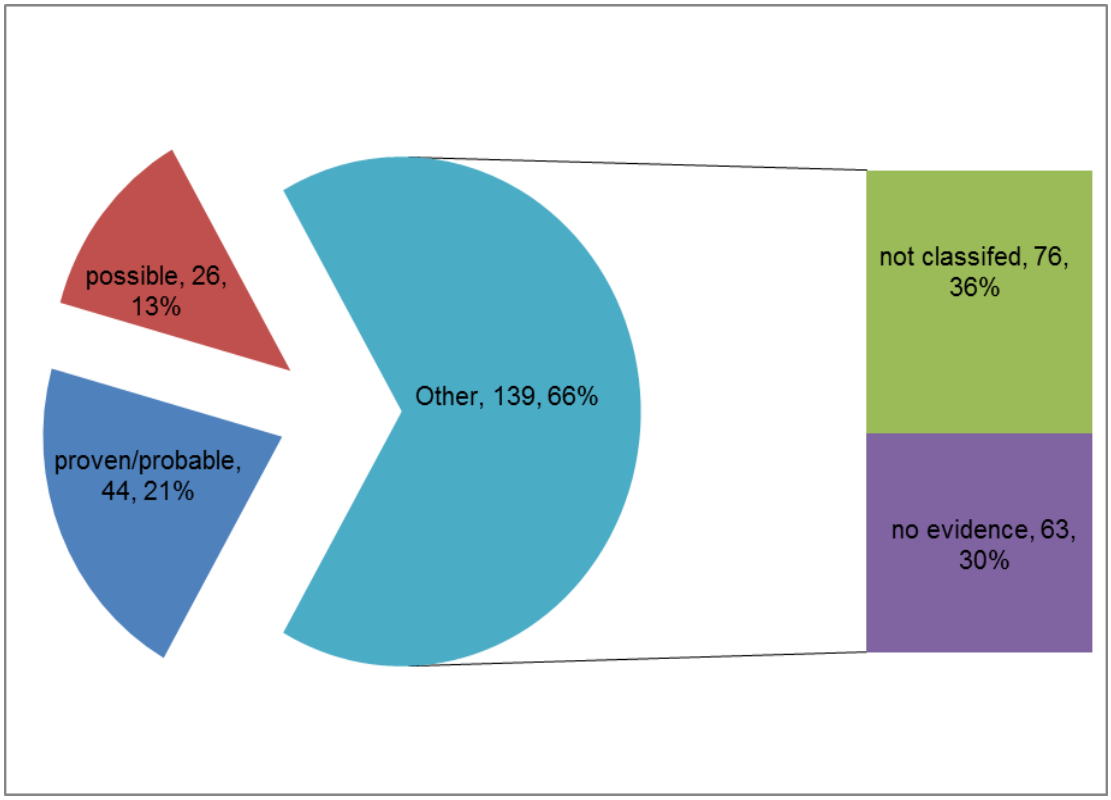


Figure 31: Study cases classified according to the revised EORTC/MSG criteria

The outcome of the EORTC/MSG classification is reliant on the diagnostic tools utilised. Using GM alone would have identified 22 probable IFDs while BDG alone would have identified 34 cases giving overall incidence of 11% and 16% respectively (Figure 32). Blood cultures identified additional three cases of *Candida parapsilosis*, *Candida guilliermondii*, and *Fusarium spp.* that were GM and BDG negative. When all the tools were utilised the number of IFD cases increased to 44 with incidence of 21%. Therefore, using GM or BDG alone would have underestimated the true incidence of IFD by 10% and 5% respectively.

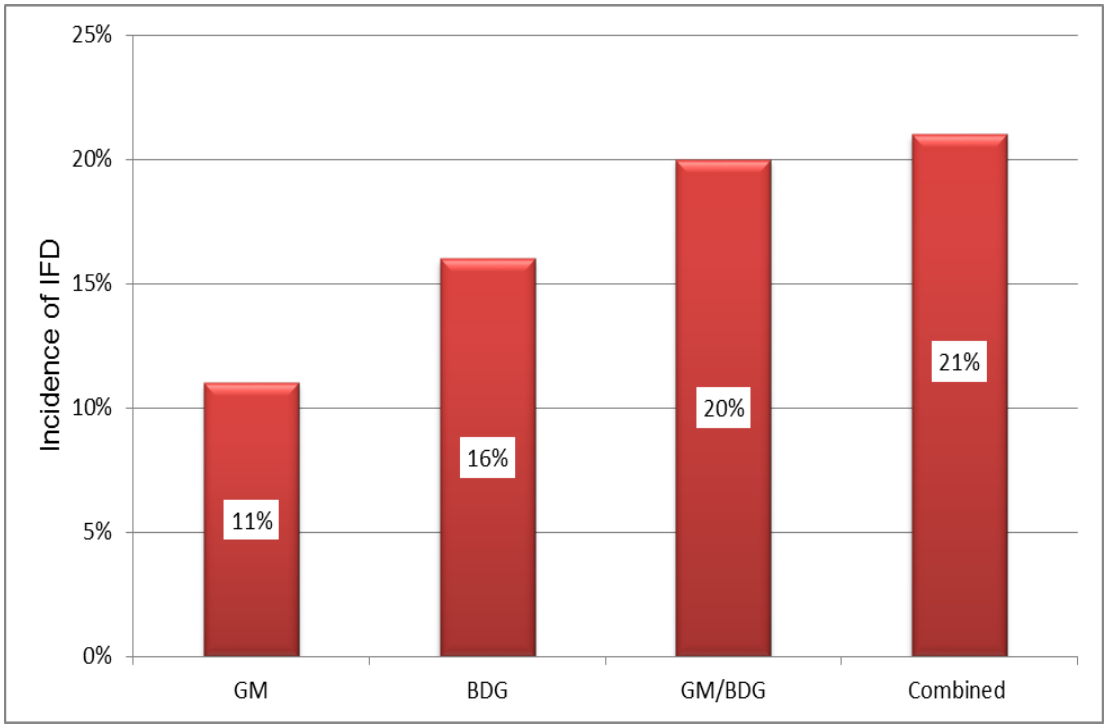


Figure 32: Incidencde of IFD varies with the diagnostic tools used

3.3.2 Treatment-specific incidences

During the course of the study the 202 evaluable patients received a total of 384 treatments for their underlying haematological malignancies (chemotherapy 177, allograft 106, autograft 72, IST 29). The index treatment, identified as the treatment during which IFD occurred, was chemotherapy (21: induction 16, consolidation 4, aplasia post consolidation 1), allogeneic HSCT (17), IST (4: pre-IST aplasia 2, IST 2) and autologous HSCT (2). Therefore, the treatment-specific incidences were 17/106 (16%), 4/29 (14%), 21/177 (12%), and 2/72 (3%) for allograft, IST, chemotherapy and autograft respectively (Figure 33).

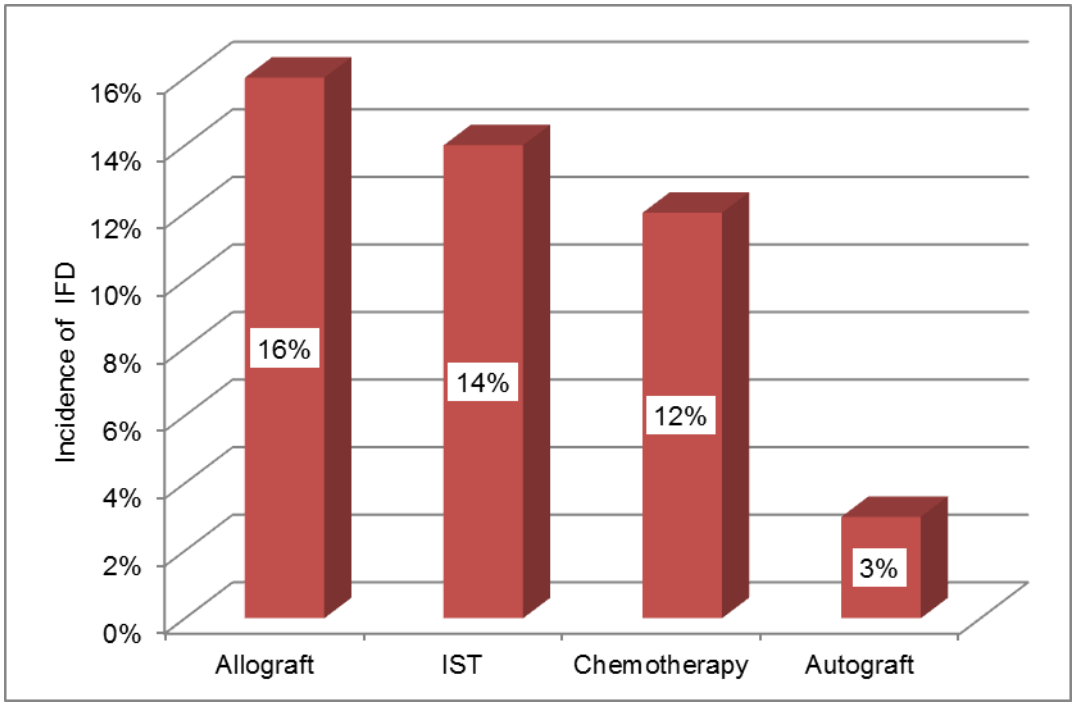


Figure 33: Treatment-specific incidence of IFD.

3.3.3 Timing of IFD

The median time from index treatment to onset of IFD was 142, 17, and 41 days for proven mould disease, candidiasis and probable IFD respectively (Figure 34). All the invasive candidiasis were detected among allogeneic HSCT patients and occurred significantly earlier than mould infection ($P<0.001$). The cumulative incidence of IFD at 3, 6, 12, and 24 months was 16%, 19%, 21%, and 21 % respectively (Figure 35). The cumulative incidence of IA at 3, 6, 12, and 24 months was 14%, 16%, 17% and 18% respectively.

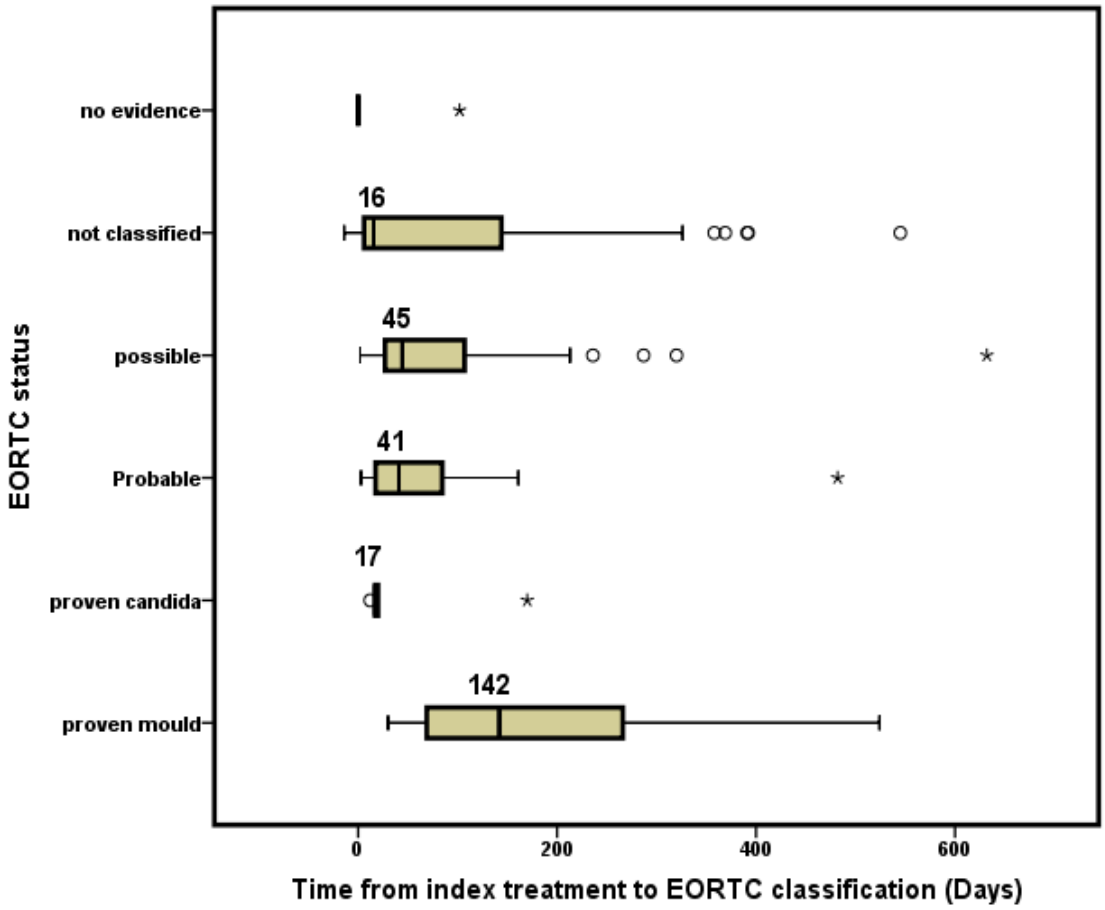


Figure 34: Interval between index treatment to EORTC/MSG classification by stem-and-leaf plot. The boxes indicate the interquartile range and the numbers on the top of the boxes indicate the median time (days) from start of study to the development of IFD.

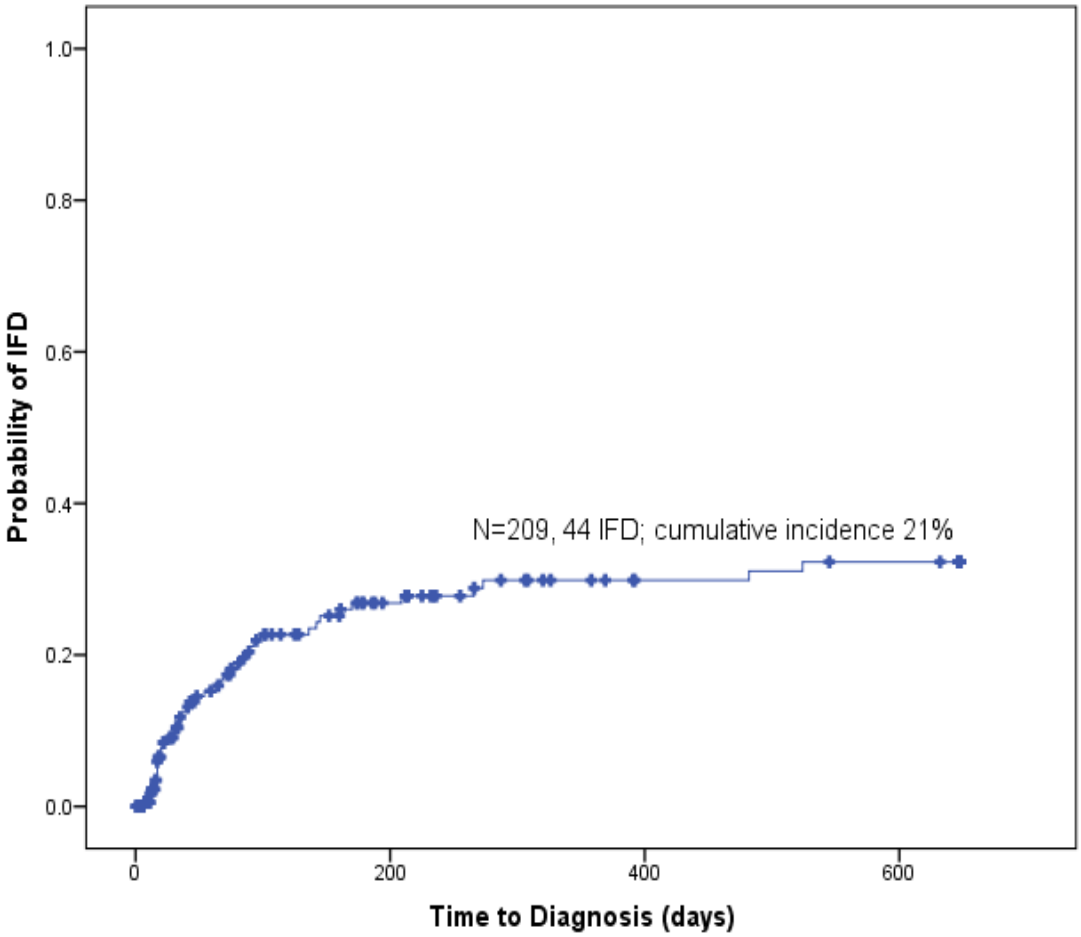


Figure 35: Cumulative incidence of IFD by Kaplan-Meir estimate

3.3.4 Patients with more than one IFD episodes

Seven patients contributed to more than one IFD episode:

1) Patient 24

   A 59y old female with aplastic anaemia probably secondary to radiotherapy she received for breast cancer 3 years prior to the aplastic anaemia diagnosis. She was admitted for IST with CSA and ATG and recruited into the study on 16/1/09. She received itraconazole as primary prophylaxis for a total of 215 days. She developed febrile

neutropenic episodes and was diagnosed with possible IPA in September 2009. She did not respond to IST and underwent a VUD allogeneic HSCT in March 2010 and grew *candida parapsilosis* from blood cultures 4 days post transplant. Her total antifungal treatment included Ambisome for 38 days, voriconazole for 85 days, and caspofungin for 44 days.

2) Patient 31

A 54y old male with refractory AML and underwent VUD allogeniec HSCT using the FLAMSA protocol. His baseline CT scan showed nodules with halo sign. Within 48h of the CT scan he developed neutropenic fever and although GM was negative BDG was positive at 523 pg/ml. He was treated with voriconazole for a total of 139 days. His follow-up CT scan at 4 weeks showed a complete radiological response. His post transplant period was complicated by grade 2 acute GVHD (cutaneous, gut and ocular) and received high dose of steroids for a total of 56 days. He also had CMV reactivations and was treated with ganciclovir for 21 days, valganciclovir for 16 days, and foscarnet for 2 days. Six months after his admission to the study, whilst on voriconazole, he developed another febrile episode and a CT scan showed mutiple nodules with halo sign. GM was positive. He was treated with ambisome for 7 days and then posaconazole for 10 days. Lung biopsy was planned but he died on the morning of the planned procedure. A coroners post mortem was carried out and cause of death was recorded as ‘fungal pneumonia’ from macroscopic appearances of

his lung lesions. No histology samples were taken as this was a coroners PM.

3) Patient 89

A 55y old male with AML for which he underwent VUD allogeneic HSCT. He developed possible IPA and *Candida guilliermondii* 12 and 17 days post allograft respectively. He received ambisome for 32 days and voriconazole for 12 days. He died from relapse 59 days after admission into the study.

4) Patient 109

A 28y old female with secondary MDS due to treatment of Burkitt lymphoma with chemotherapy and total body irradiation 9 years previously. She was admitted for standard cord blood allogeneic HSCT following inadequate response to 6 cycles of 5-azacytidine. She received posaconazole as primary prophylaxis for 25 days. Her baseline CT scan showed nodules in the lingula and left lower lobe (Figure 36). She was clinically well with no fever, or any other signs of sepsis. GM and BDG were negative. FBC showed Hb 8.9 g/dl, WBC 1.64 $x10^9$/L, neutrophils 0.26 $x10^9$/L, lymphocytes 1.33 $x10^9$/L, monocytes 0.02 $x10^9$/L, and platelets 61 $x10^9$/L. CRP was <5mg/l. Three days after the baseline CT scan she had her first of five febrile episodes and was started on first line antibiotics. She failed to respond to second line antibiotics during her 3rd febrile episode and had a diagnostic scan. This showed nodules in the RUL, LUL, RML, LLL and a cavity in the LLL (Figure 36). GM and BDG taken 2 days prior to this febrile episode were

positive with GMI of 1.44 and BDG of 282 pg/l. She was treated with ambisome for 14 days before being changed to posaconazole. Lung biopsy using the VATS approach 10 days after the diagnostic scan showed invasive mould infection NOS. She had 2 further febrile neutropenic episodes and grew *candida kefyr* during one of these. She died from EBV-driven post-transplant lymphoproliferative disorder (PTLD) classical Hodgkin lymphoma subtype 192 days after admission into the study. This presented as a febrile episode resistant to second-line antibiotics while she was already on treatment dose of ambisome. The CT scan showed bilateral nodules in the RUL, LUL, lingula, RML, RLL, and LLL. Despite chemotherapy for her PTLD she deteriorated and died in haematological remission.

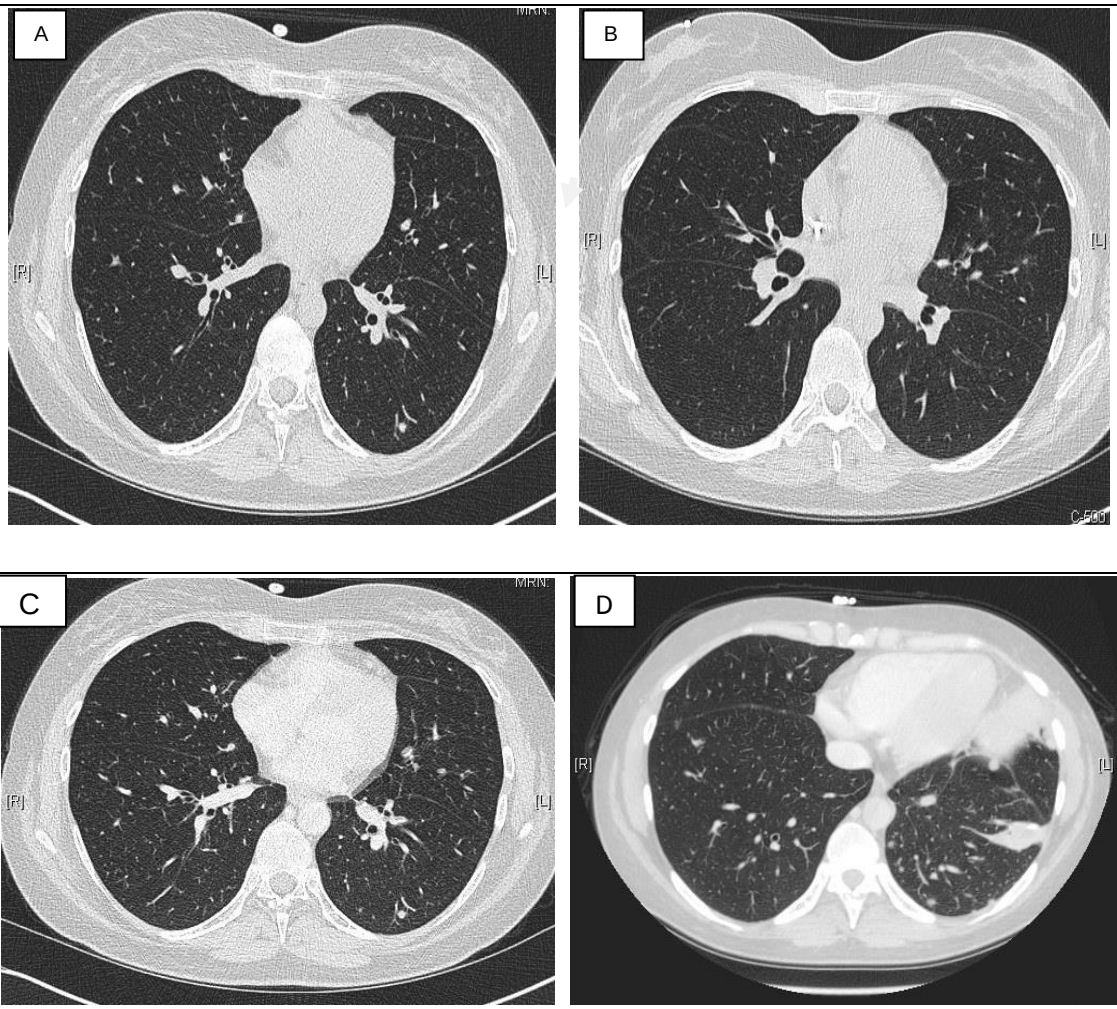


Figure 36: Patient 109. Baseline CT scan (A) showing a nodule with halo in the lingula. She was well and CRP was <5mg/l. No antifungal treatment was initiated. Diagnostic scan was performed 26 days after baseline scan without contrast (B) and with contrast (C) showing LLL nodules and both GM and BDG were positive. VATS biopsy confirmed invasive mould disease NOS. Second diagnostic scan (D) showing multiple nodules. Lung biopsy showed PTLD.

5) Patient 127

A 54y old male with MDS/MPD admitted for induction chemotherapy in August 2009. He had a total of 11 septic neutropenic episodes in 85 days during 492 study days. He had total of 389 days of prophylaxis with itraconazole and 91 days of treatment with Caspofungin (20 days), Ambisome (12 days), and voriconazole (59 days). During his first

neutropenic sepsis in September 2009 he did not respond to second-line antibiotics and CT showed a nodule in his LLL. GM was negative (GMI 0.14) but BDG was positive at 137 pg/ml. He was therefore diagnosed with probable IFD. He had further febrile episodes in November 2009 when pulmonary CT showed RUL nodule and BDG was positive. He was treated with caspofungin and his follow up CT scan showed a complete radiological response.

In October 2009 he was admitted for a matched sibling allogeneic HSCT with FLAMSA approach. He relapsed post allograft and had to have re-induction chemotherapy followed by a second allograft in October 2010. During this period he had further neutropenic sepsis and CT scan showed a mass with halo sign in the LLL. GM remained negative but BDG was again positive. He then underwent a VATS biopsy which showed invasive mould disease. Fungal cultures were negative. He subsequently relapsed from his MDS/MPD and died.

6) Patient 128

A 43y old female with MDS/Fanconi anaemia and admitted for cord blood allogeneic HSCT and was recruited into the study in August 2009. She had a total of 8 febrile neutropenic episodes lasting for a total of 145 days during her study period of 469 days. During this period she had 269 days of antifungal prophylaxis (itraconazole 31 days, ambisome 38 days, posaconazole 200 days) and 224 days of antifungal

treatment (ambisome 28 days, caspofungin 28 days, voriconazole 5 days, and posaconazole 163 days). Her baseline CT prior to her transplant showed nodules and halo sign in the RUL and lingula, and a mass and cavity in the RUL. She was clinically well and underwent VATS biopsy which showed non-specific interstitial pneumonitis. In October 2009 she was diagnosed with probable IPA with nodules and halo sign associated with multiple positive GM and BDG results. VATS biopsy showed non-specific inflammatory changes probably due to inadequate samples obtained. In April 2010 during her 6th neutropenic sepsis episode, despite antibiotics and antifungal therapy, she continued to have fever. Bronchoscopy was performed and the BAL showed *pneumocystis jiroveci* on Grocott staining.

7) Patient 164

46y old man diagnosed with MDS/MPD who was admitted for induction chemotherapy in November 2009 followed by VUD allogeneic HSCT in January 2010. He experienced 2 febrile neutropenic episodes in December 2009 and November 2010. The CT scan in both cases showed bilateral nodules with halo signs. Both GM and BDG were negative throughout and he was therefore classified as having two possible IPA episodes. He was in complete radiological remission from his 1st IPA at the week 4 scan.

3.3.5 Concomitant bacterial and viral infections

Bacterial infections, where organisms were recovered from specimen cultures, were seen in 74 (36%) of this cohort. These organisms were Gram positive cocci (27; 36%), Gram negative rods (25; 34%), mixed (20; 27%) and non-tuberculous mycobacterial spp (2; 3%). Concomitant bacterial infections, where organisms were recovered during the same febrile episode as the EORTC/MSG diagnosis, were seen in 18 (8.9%) of the study patients (Table 20). Among the proven/probable IFD cases concomitant bacterial infections were seen in 8 (4.0%) patients. Two organisms were cultured during the febrile episode from 4 of these 8 concomitant infections.

Viral infections/reactivations were seen in 24 of the 99 allograft patients (24%): CMV in 13, CMV/adenovirus in 2, CMV/EBV in 1, EBV in 5, adenovirus in 2, adenovirus/BK virus in 1. Viral reactivations detected by PCR were only monitored in allograft patients.

Table 20: Clinical and laboratory characteristics of the 18 cases of concomitant bacterial infections

EORTC class	Diagnosis	Treatment	GM results	BDG results	Organisms
Proven, n=3 *A. fumigatus* (2), Mould NOS (1)	ALL, CLL, MDS/FA	Chemo (1), allo (2)	Pos (2)	Pos (2)	*Enterococcus faecalis Pseudomonas aeroginosa , Diphtheroids, stenotrophomonas maltophilia , VRE*
Probable, n=5	AA (1), AML (1), MDS (2), HD (1)	Allo (2), auto (1), chemo (1), IST (1)	Pos (4)	Pos (4)	*Mycobacterium cheloni, Klebsiella spp, Diphtheroids, VRE*
Possible, n=1	AML	Allo	Neg	Neg	*Micrococcus spp*
Not classified, n=8	Myeloma (3), AA (2), NHL (2), AML (1)	Allo (3), auto (4), IST (1)	Pos (6)	Pos (6)	*E. coli, pseudomonas aeroginosa, Klebsiella spp, Acinetobacter baumannii, VRE, staph aureus*

3.4 Antifungal agents

3.4.1 Prophylaxis

Itraconazole and fluconazole were the most commonly used prophylactic agents for high and low risk patients respectively according to local antifungal guidelines 2007 (Table 21). However, there was a significant deviation from protocol for posanacozole, voriconazole and ambisome. Itraconazole was admistered intravenously in 15/215 (7%) of cases and in the rest as a solution/suspension. The median duration of antifungal prophylaxis was 87 days (interquartile range 36-164 days). Eleven patients received no prophylaxis as they were receiving emperic antifungal treatment (9) or none (2) at the time of recruitment. The latter two patients were admitted for allogaft which were subsequenly cancelled.

Table 21: Antifungal agents used for prophylaxis according to KCH antifungal protocol 2007

Antifungal agent	Unit protocol-primary prophylaxis N (%)	Unit protocol-secondary prophylaxis N (%)	Not protocol N (%)
Itraconazole N=215	192 (89)	0 (0)	23 (11)
Fluconazole N=59	56 (95)	0 (0)	3 (5)
Posaconazole N=68	1 (2)	15 (22)	52 (76)
Voriconazole N=23	1 (4)	5 (22)	17 (74)
AmBisome N=30	5 (17)	11 (37)	14 (47)

3.4.2 Treatment

Antifungal therapy was given to 101 (50%) patients during the course of the study for suspected IFD (Figure 37). The median duration of treatment was 32 days (interquartile range 8-80 days; range 1-456 days). Patients who were treated for 2 weeks or more (33%) was similar to the combined incidence of proven/probable/possible IFD (70/209 33%) and also the Chamilos PM data.[77]

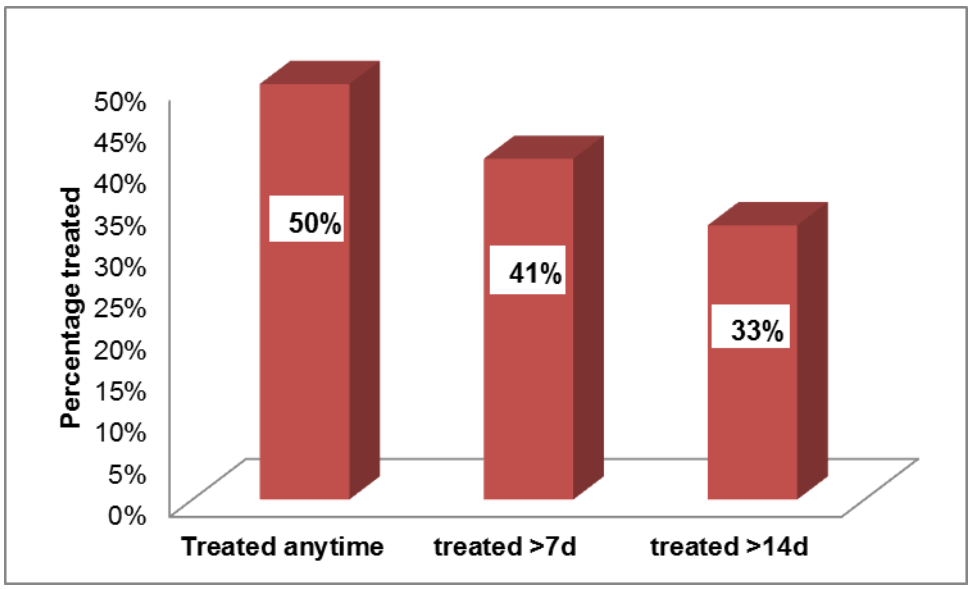


Figure 37: Proportion of patients treated with antifungal agents for suspected IFD during the course of the study.

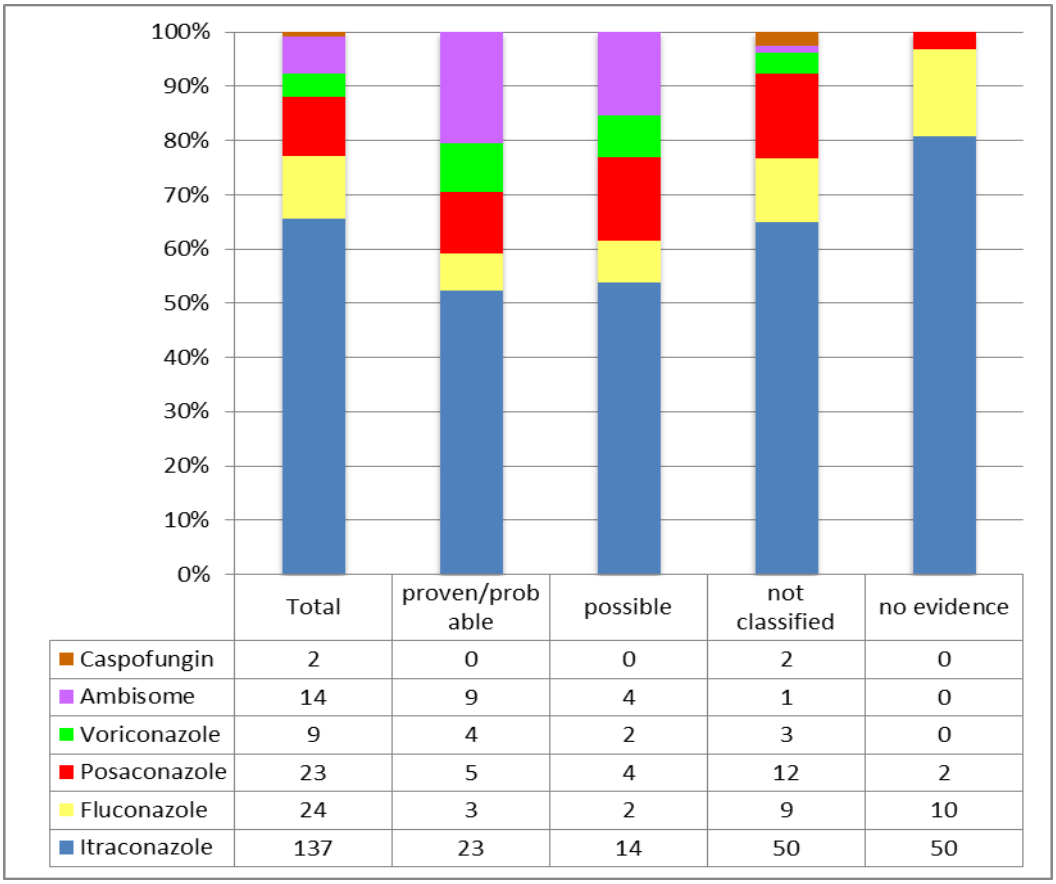
In total 266 treatment episodes were adminstered using the drugs shown in Table 22 according the KCH antifungal guidance 2007 or ITU admission. 62 of the 101 (61%) Ambisome treatments were as first line agent compared to voriconazole where 34/41 (83%) were given after initial therapy with ambisome or caspofungin.

Table 22: Antifungal treatments used according protocol or ITU admission

Antifungal agent	Unit protocol	ITU	Other
Posaconazole, N=50	37 (74)	1 (2)	12 (24)
Voriconazole, N=41	33 (81)	1 (2)	7 (17)
Ambisome, N=101	78 (77)	0 (0)	23 (23)
Caspofungin, N=74	45 (61)	3 (4)	26 (35)

3.4.3 Antifungals at time of IFD diagnosis

Itraconazole was the most commonly used antifungal drug, used in 65% of patients, within 2 weeks of the EORTC/MSG classification (Figure 38). Based on these the break-through proven/probable IFD was 5/23 (22%), 23/137 (17%), and 3/24 (13%) for posaconazole, itraconazole, and fluconazole, respectively. Ambisome, caspofungin, and voriconazole were used as empiric treatment. Because itraconazole, posaconazole and fluconazole were used in different risk strata, these break-through rates are not directly comparable.



	Total	proven/probable	possible	not classified	no evidence
Caspofungin	2	0	0	2	0
Ambisome	14	9	4	1	0
Voriconazole	9	4	2	3	0
Posaconazole	23	5	4	12	2
Fluconazole	24	3	2	9	10
Itraconazole	137	23	14	50	50

Figure 38: Antifungal drugs patients used within 2 weeks of EORTC/MSG classification

3.4.4 Prophylaxis-treament patterns among proven/probable IFDs

Among proven/probable IFDs the commonest pattern of prophylaxis-treatment was the use of azole as primary or secondary prophylaxis followed by either Ambisome or caspofungin [azole-other] (Figure 39). Azole-other combination was used in 31 of the 44 (68%) proven/probable IFD cases (Table 23). Azole prophylaxis followed by azole treatment [azole-azole] was uncommon in this cohort with only 4 cases seen among the proven/probable cases.

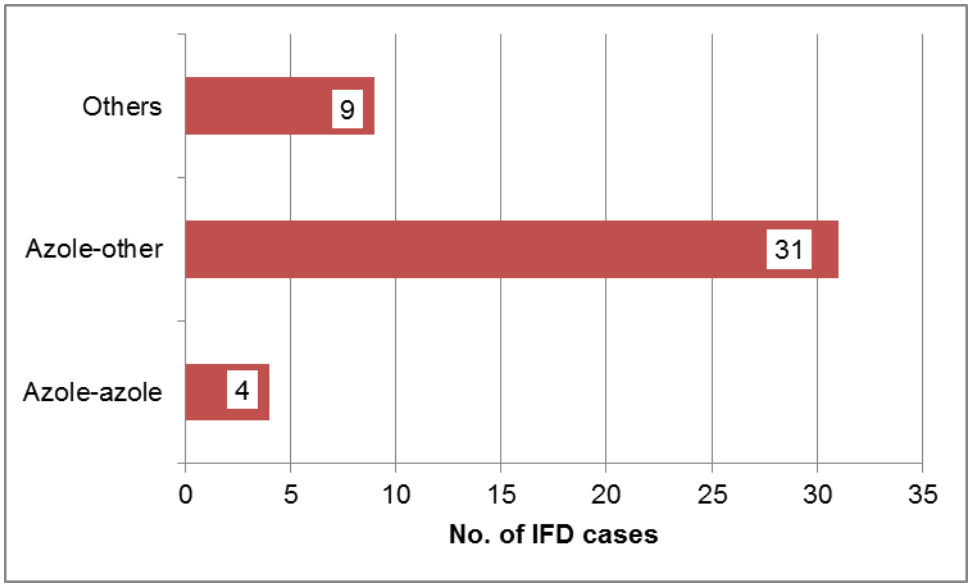


Figure 39: The pattern of prophylaxis to treatment among the 44 proven/probable IFDs

Table 23: Pattern of prophylaxis-treatment among proven/probable IFD cases

	Combination	No. of cases
Azole-Azole	Itraconazole-voriconazole	1
N=4	Itraconazole-posaconazole	2
	Posaconazole-voriconazole	1
Azole-other	Itraconazole-ambisome	12
N=31	Itraconazole-caspofungin	6
	Posaconazole-ambisome	4
	Posaconazole-caspofungin	2
	Voriconazole-ambisome	3
	Voriconazole-caspofungin	1
	Fluconazole-ambisome	3
Others	None-ambisome	5
N=9	Ambisome-ambisome	3
	Itraconazole-none	1

3.5 Galactomannan and β-D-glucan performance

3.5.1 Galactomannan

Using the programmed surveilance method described in Chapter 2, 3086 serum samples were tested from the study patients. The median number of samples per patient was 11(range 1-90). The mean GMI was 0.22 (range 0.01-8.3, IQR 0.09-0.22) and was significantly higher at 0.32 in the chemotherapy only group vs 0.20, 0.19 in the allograft and autograft groups respectively (P<0.001). Using GMI cut off of ≥0.5, 169 (5%) samples were positive (Figure 40). The chemotherapy-only group had a significantly higher proportion positive results at 9.6% vs 4.7% and 4.3% in the allograft and autograft groups respectively (P <0.001).

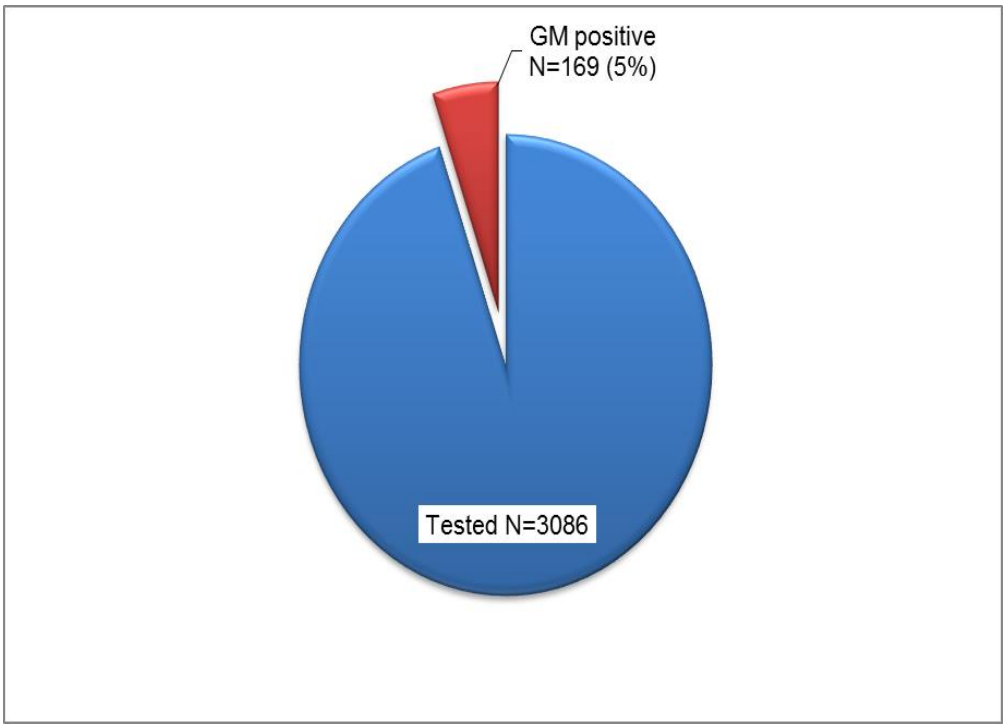


Figure 40: Serum galactomannan testing on 3086 samples and proportion of positive results (GMI ≥0.5)

Among the 169 positive GM samples 62 (37%) were from 22 proven/probable IFD cases representing 2% of the total samples tested and the rest were unclassified. In 10 of the 22 cases (45%) GM positivity preceeded CT signs. Eleven of these 62 GM positive samples were considered false positives as they were unrelated temporally to the IFD episode. Therefore, the false positive rate among the proven/probable cases was 18% (11/62). The sensitivity, specificity, PPV, and NPV were 54% (49/91), 71% (27/38), 82% (49/60), and 39% (27/69), respectively.

3.5.2 Does persistent GM positivity predict poor prognosis

The 22 probable/proven cases identified as GM positive occurred in 21 patients as one of them had 2 separate episodes (See section 3.3.3). Eight of these patients had ≥2 GM positive results (Figure 41). Six of the patients remained persistently positive and all died with active IPA. Patient 1 relapsed from his AML as his IPA worsened. Patient 3 was particularly intrigiung. She was a 47 year old telephone operator diagnosed with lymphoblastic lymphoma and was admitted for induction chemotherapy on the UKALL 12 protocol. She had 4 separate febrile neutropenic episodes for a total of 50 days and was treated with broad-spectrum antibiotics and received ambisome as primary prophylaxis as per protocol. Her GM was positive on 13 different occations, 4 of which occurred 66, 64, 60, 11 days prior to the EORTC/MSG diagnosis with GMI of 0.75, 0.51, 0.97 and 0.75 respectively. CT scan carried out as a result of the second positive GM results (she was afebrile at the time) was completely normal. She was subsequently diagnosed with probable IPA and in

total received 83 days of antifungal therapy (ambisome 48 days, caspofungin 26 days, posaconazole 9 days). Despite this her GMI continued to increase (Figure 41) in parallel with clinical deterioration. She subsequently died and had a coroners PM and cause of death was recorded as 'bronchopneumonia' but no histology samples were taken due to the nature of the PM.

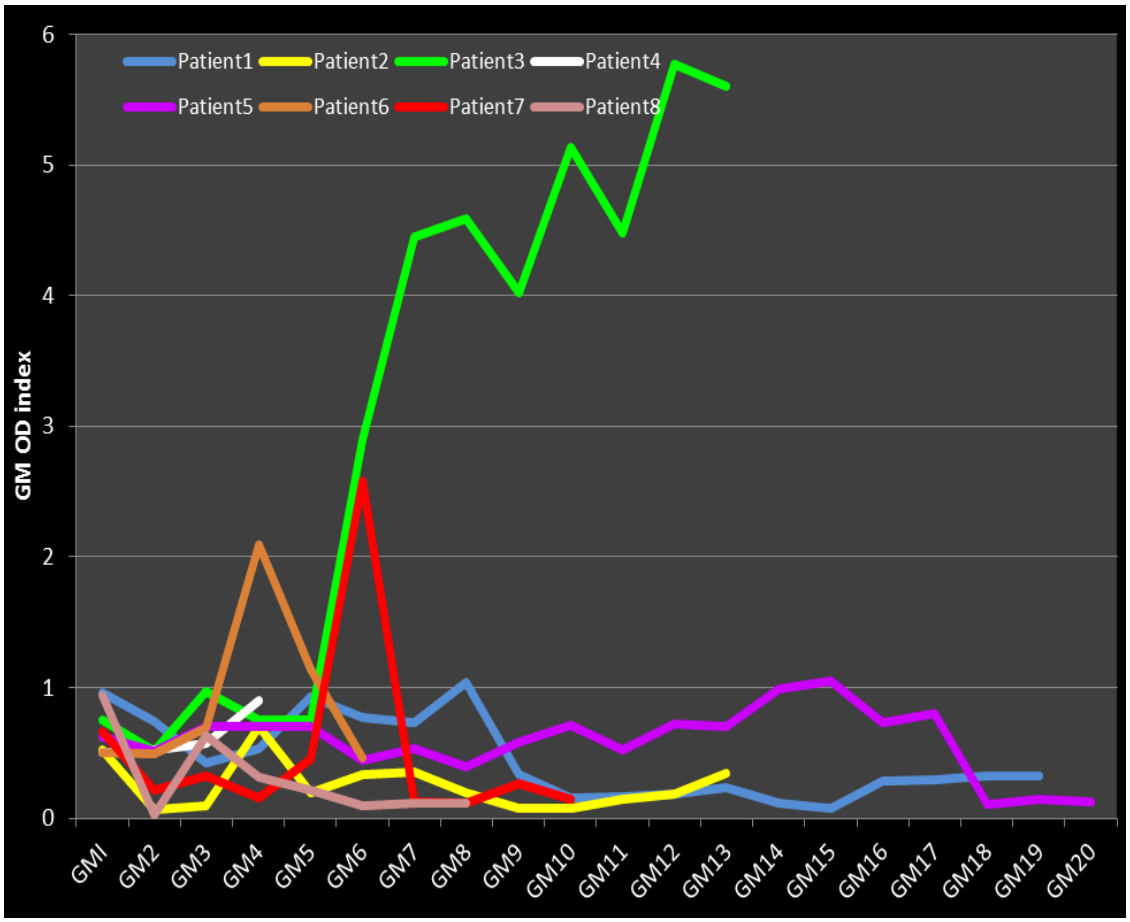


Figure 41: Quantification of Galactomannan antigenaemia by ELISA in 8 patients with proven/probable IPA who had ≥2 GM positive readings. Patient 1 died from relapsed AML with active IPA, patient 2-6 died from IPA. Patients 7 & 8 cleared GM antigen and achieved CR of IPA and are alive.

3.5.5 β-D-glucan

A total of 253 samples from 179 patients were tested of which 152 were positive (>80pg/ml), 18 were indeterminate (60-79 pg/ml) and 83 were negative (<60 pg/ml). In this analysis, indeterminate results were considered negative and therefore 101 samples were negative. The mean BDG concentration was 147 pg/ml (95% CI 130-164) and this was significantly lower in the autograft patients at 90 (95% CI 63-117) vs allograft and chemotherapy groups at 139 (95% CI 120-159) and 198 (153-243) respectively (P<0.001; Table 16). The proportion of samples with positive BDG results was 60% and this was not significantly different between the groups (P= 0.079). The sensitivity, specificity, PPV, and NPV were 79% (66/84), 55% (17/31), 83% (66/80), and 49% (17/35), respectively.

3.5.6 Relationship between GM and BDG

Serology testing with GM and BDG was positive in 40 of the 44 proven/probable cases. Two cases of candida and one *fusarium* were negative while no sample was available from the cardiac aspergillosis case at the time of diagnosis. Both GM and BDG were positive in 15 of the 40 IFD cases (Figure 42). There was no significant differences between the GM and BDG positive proven/probable cases in terms of underlying haematological diagnosis (P=0.442), treatment group (P=0.227), and survival (P=0.816).

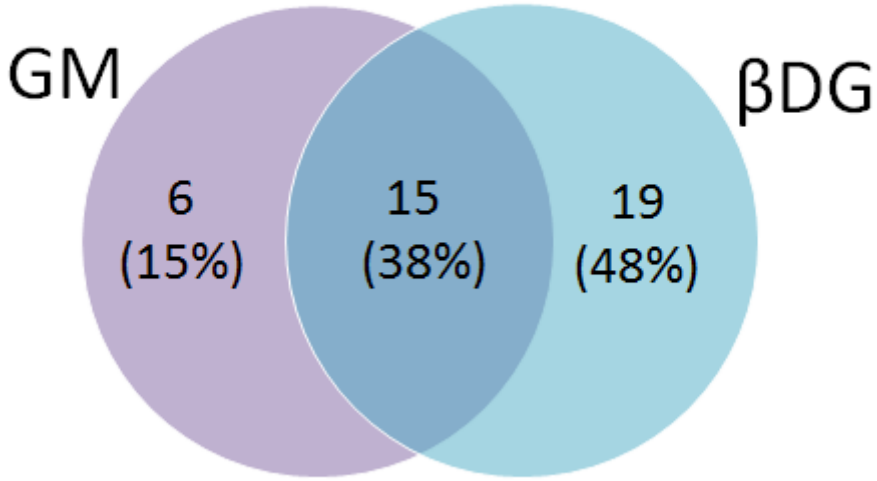


Figure 42: Relationship between BDG and GM positive results among 40 of 44 proven/probable IFD serology-positive cases.

3.6 LFD

A total of 973 sequential serum samples from 56 patients were tested with LFD (Table 24). There were 35 positive samples (3.6%; strongly positive 10, moderately positive 11, weak positive 14) from 7 patients (proven mould NOS 1, probable IFD 4, and 2 false positives). The 2 false positives were a possible IFD and proven *A. fumigatus* where the LFD positivity preceed the EORTC diagnosis by 170 and 38 days respectively. Therefore, using LFD as the sole mycological diagnsotic tool in this cohort the inicdence of IFD would be 8.9% (5/56).

Table 24: Results of 973 sera tested with LFD and GM and a selection for BDG.

	LFD, n (%)	**GM, n (%)**	**BDG, n (%)**
Aspergillus fumigatus	0/5 (0)	3/5 (60)	2/2 (100)
Moulds NOS	7/71 (10)	4/71 (6)	5/6 (83)
Fusarium spp	0/4 (0)	0/4 (0)	0/1 (0)
PCP	0/18 (0)	0/18 (0)	-
Candida spp	0/12 (0)	0/12 (0)	1/1 (100)
Probable IFD	22/250 (9)	39/250 (16)	41/48 (85)
Possible IFD	1/73 (1)	2/73 (3)	2/7 (29)
Not classified	0/246 (0)	38/246 (15)	26/46 (57)
No evidence	5/294 (2)	0/302 (0)	6/10 (60)

The modified LFD method with pre-treatment of sera was performed on 16 samples from one of the patients with proven pulmonary *A. fumigatus*. Only one sample was weakly positive 24 and 38 days prior to possible/probable and proven IFD, respectively. These results were the same as the none pre-treated ones and therefore no further pre-treatment tests were performed.

The true performance of the tests is best assessed by the results of individual samples. The true disease status was defined as proven aspergillosis, mould NOS and probable IFD. Patients with no evidence of IFD, candida, fusariosis, and PCP were considered true negatives. Possible IFD and not classified categories were excluded. Restricting the true disease status only to GM-positive cases the sensitivity, specificity, PPV, and NPV of LFD was 4/46 (9%), 311/316 (98%), 4/9 (44%), and 311/353 (88%) respectively.

Relationship between LFD and GM and BDG

Using LFD and GM as diagnostic tools identified 23 cases of proven/probable IFDs. Only 2 patients were identified by both tests and 3 were identified only by LFD (Figure 43). A similar number of patients were identified by LFD and BDG but no additional cases were identified by LFD while five patients were identified by both tests (Figure 44).

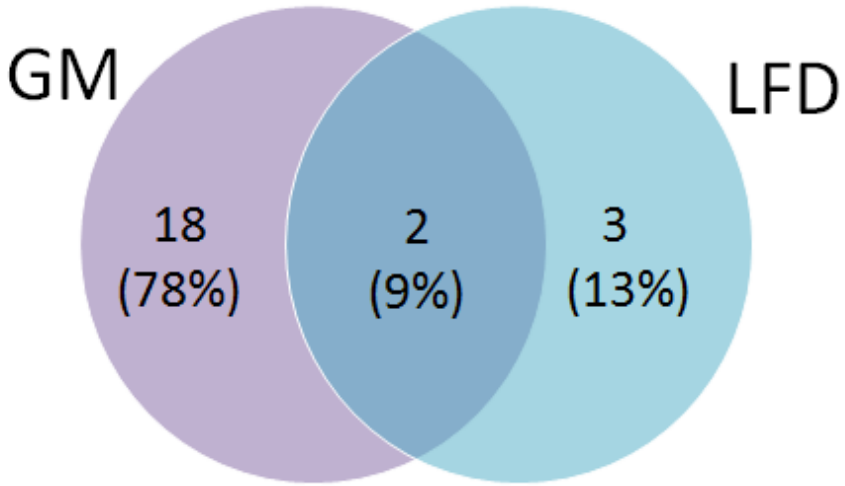


Figure 43: Relationship between positive LFD and GM in identifying IFD cases

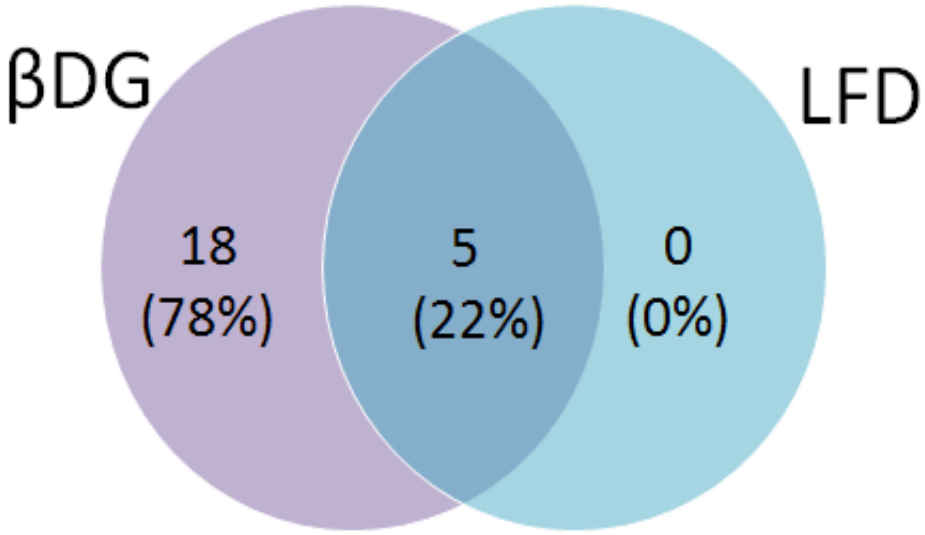


Figure 44: Relationship between positive LFD and BDG in identifying IFD cases

3.7 PCR

323 blood (in EDTA) samples from 52 patients were tested. Of these 19 (3%) samples were positive (Ct< 50) from 11 patients. The Ct of the positive samples ranged from 36-49 suggesting weak to moderate positivity. The 11 patients included those with *A. fumigatus* (1), mould NOS (1), probable IFD (1), possible IFD (1), not classified (2), no evidence (3) and 2 false positives. The false positives were a case of *Candida glabrata* and a probable IPA where PCR was positive 8 and 3 weeks respectively prior to the EORTC/MSG diagnosis. Therefore, PCR truly identified 3/52 cases (5.8%). The sensitivity, specificity, PPV, and NPV were 15% (9/60), 97% (138/143), 75% (9/12), and 73% (138/189), respectively for IFD. The corresponding values for IA were 14% (8/59), 97% (138/143), 62% (8/13), and 73% (138/189). All the true positive PCR results were also GM positive.

PCR was also carried out on 96 serum samples (proven *A.fumigatus* (6), proven mould NOS (10), probable (32), possible (2), not classified (24), no evidence (22)) from 16 patients. There were 21 (21.9%) positive samples from 10 patients with A. fumigatus (4), mould NOS (6), probable IFD (7), possible (1), not classified (2), no evidence (1) . Two of the probable cases were likely to be false positive as they occurred 16 days after (while on antifungal therapy) and 4 weeks prior to the EORTC/MSG classification respectively. The sensitivity, specificity, PPV, and NPV of serum PCR was 35% (17/48), 96% (21/22), 94% (17/18), and 40% 921/52), respectively. There was little correlation between blood and serum PCR as only 5 of the 21 were positive by

both methods (Figure 45). These samples were from 2 patients with proven *A. fumigatus* and mould NOS.

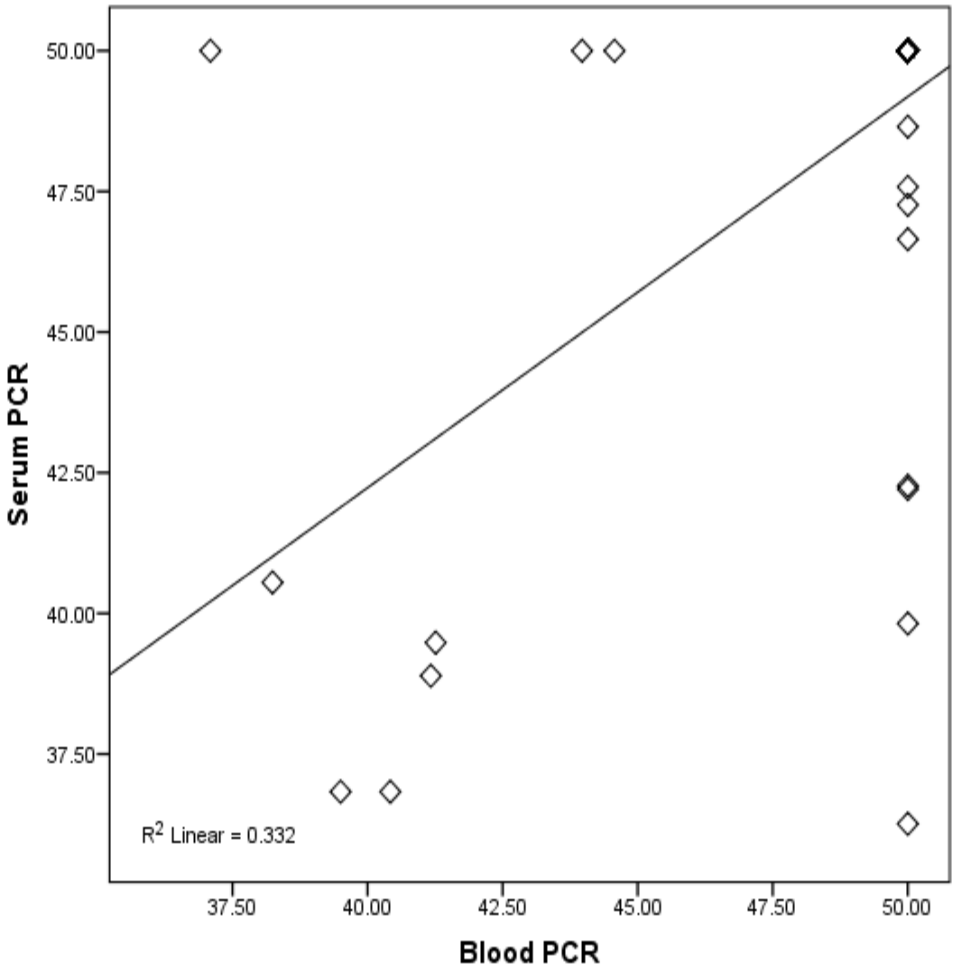


Figure 45: Scatter plot of PCR on blood and serum samples. Only 5 samples were positive (Ct <50) by both methods. The numbers reflect the Ct values

3.8 Bronchoscopy

Broncho-alveolar lavage (BAL) was performed on 9 patients. Three of these were pre-conditioning baseline procedures and 9 were diagnostic requests based on clinical indications (Table 25).

Table 25: BAL results

Case	Diagnosis	Treatment	Final EORTC status	Request type	GM	Virology	Bacteriology	Cytology
1*	MDS	Allograft	Probable	Baseline	Neg	-	Neg	Neg
2*	MDS	Allograft	Probable	Diagnostic	Neg	Neg	*P. aeroginosa*	Neg
3	Myeloma	Autograft	NE	Baseline	Neg	Neg	Neg	ND
4†	MDS	Allograft	NE	Baseline	Neg	Neg	Neg	Neg
5†	MDS	Allograft	NE	Diagnostic	Neg	Neg	Neg	ND
6	MDS	Chemo	NC	Diagnostic	Neg	Neg	Neg	Neg
7	MDS	Chemo	NC	Diagnostic	Neg	Neg	Neg	Neg
8	MDS	Allograft	Probable	Diagnostic	Neg	Neg	*K.pneumoniae*	Neg
9	ALL	Chemo	Proven	Diagnostic	Neg	Neg	Neg	Neg
10	AA	Allograft	Possible	Diagnostic	Neg	Neg	Neg	Neg
11§	MDS/FA	Allograft	Proven	Diagnostic	Neg	Neg	Neg	*Pneumocystis jiroveci*
12§	MDS/FA	Allograft	Proven	Diagnostic	-	CMV	Neg	Neg
13§	MDS/FA	Allograft	Proven	Diagnostic	-	CMV	CNS	Neg

* †§ represent the same group of patients

Abreviations: CNS, coagulase negative staphylococci; not classified; NE, no evidence; NC, ND, not done.

3.9 Biopsy

3.9.1 Patient characteristics and clinical features

Table 26 shows the characteristics of the 38 cases biopsied. The main clinical features at the time of CT were fever (84%), abnormal baseline CT without fever (11%), and lack of adequate response to antifungal therapy (5%). Extra-pulmonary presentation was seen in 4 cases: mass in the hard palate (2), mediastinal mass with pulmonary lesions (1) and cardiac mass with vegetations (1). The median neutrophil count was 1.5 $x10^9$/L (range 0.02- 7.35 $x10^9$/L) and 17 (45%) cases had neutrophils <0.5 x 10^9/L at time of biopsy. The median duration of neutropenia prior to CT was 16 days (range 0- 233 days). The median pre-biopsy haemoglobin and platelet counts were 9.5 g/dl (range 6.9- 13.1 g/dl) and 113 $x10^9$/L (range 45- 399 $x10^9$/L) respectively. There were pre-operative transfusions in 30 (79%) cases.

The primary pulmonary radiological features were: nodules in 32 cases (84%), nodules with halo sign in 19 cases (50%), nodules with cavity in 2 cases (5%), and consolidation in 5 cases (13%).

Table 26: Characteristics of biopsy cases

Characteristics	Values
Age, y, Median (range)	43 (21-73)
Sex, M:F ratio	19:19
Diagnosis, n (%)	
Acute Myeloid Leukaemia	8 (21)
Myelodysplastic syndromes	11 (29)
Aplastic anaemia	6 (16)
Hodgkin lymphoma	5 (13)
Non-Hodgkin lymphoma	3 (8)
Others*	5 (13)
Treatment received, n (%)	
Allogeneic HSCT	19 (50)
Autologous HSCT	5 (13)
Chemotherapy	14 (37)

Patients with more than one episode of biopsy were counted more than once.

*others included ALL 2, multiple myeloma 1, CLL 1, common variable immunodeficiency syndrome 1.

3.9.2 Histological findings

The results of the biopsy were divided into groups A, B, and C (Table 27). The diagnosis of IFD was confirmed in 7 cases (18%) (Group A). *A. fumigatus* was recovered by culture from two of these cases. One of these was a 59 year-old woman with CLL who developed a sub-aortic valve and ventricular mass invading into the myocardium at day 258 post-matched unrelated donor transplant using alemtuzumab-based conditioning regimen on immunosuppressive therapy for chronic graft-versus host disease (see case 7 section 3.3). The second was a 67 year-old man with ALL undergoing induction chemotherapy and developed IPA. The other five cases in group A

were two cases of hard palate and three cases of culture-negative pulmonary mould IFD.

An alternative diagnosis was obtained from 7 cases (18%) [Group B]. Two cases of these were Hodgkin lymphomas: an EBV-driven post-transplant lymphoproliferative disorder (PTLD) in a female post umbilical cord HSCT for secondary MDS and a relapsed nodular sclerosis Hodgkin lymphoma. The other five cases were chronic GVHD of the lungs (2), sclerosing haemangioma (1), multiple pulmonary embolism (1) and mediastinal tuberculous adenitis (1) due to *Mycobacterium kansasii* post umbilical cord HSCT.

The remaining patients (24, 63%) were grouped under a miscellaneous category in group C. These were all lung biopsies and methylene blue was used in 4 of these procedures to aid pre-operative localisation. This group included cases where the predominant histological pattern was inflammatory changes but fungal hyphae were not seen (9), diffuse alveolar damage of uncertain aetiology (9), non-specific changes (3), and normal lung tissue (3). The mean (95% CI) interval between CT and biopsy was 16 days (11-21 days). This was significantly longer in group B with 34 days compared to 15 and 11 days for groups A and C respectively (P=0.001). The pre-biopsy radiological features and EORTC classification was similar between the groups (Table 2). Overall, biopsy results were useful in 14 cases (37%) where the diagnosis was proven (group A) or an alternative diagnosis was made (group B).

Table 27: Results of the biopsies and associated characteristics

Characteristics	Group A: IFD N= 7	Group B: alternative diagnosis N= 7	Group C: miscellaneous N= 24	P value
Histology, n	*A. fumigatus,* 2 Mould IFD, 5	Chronic GVHD, 2 Hodgkin lymphoma, 2 Sclerosing haemangioma, 1 PE, 1 TB adenitis (*M.kansasii*), 1	Inflammatory changes, 9 Diffuse alveolar damage, 9 Non-specific, 3 Normal, 3	-
Site of biopsy	Lungs (4) Palate (2) Heart (1)	Lungs (6) Mediastinum (1)	Lungs (24)	0.004
Age (median), y	47	39	46	0.527
Adequate sample, n (%)	7 (100)	7 (100)	8 (33)	<0.001
Treatment, n (%)				
Allogeneic HSCT	4 (57)	5 (71)	10 (42)	0.197
Autologous HSCT	0 (0)	0 (0)	5 (21)	
Chemotherapy	3 (43)	2 (29)	9 (37)	
CT to biopsy, Median interval, days	15	34	11	0.001
CT signs, n (%)				
Nodules	4 (100)	5 (83)	21 (88)	0.133
Halo sign	3 (75)	2 (33)	14 (58)	0.062
Cavity	0 (0)	1 (17)	1 (4)	0.053
Pre-biopsy, n (%)				
Possible	0 (0)	2 (29)	13 (54)	0.124
Probable	6 (86)	2 (29)	8 (33)	
Not classified	1 (14)	3 (42)	3 (13)	
Median duration of AF prophylaxis, d	86	23	82	0.337
Median duration of AF treatment, d	135	40	77	0.154
Neutropenia, n (%)	3 (43)	4 (57)	10 (42)	0.166
Median duration of neutropenia, d	64	18	40	0.268

The inflammatory changes included cases where IFD could not be excluded and included granulomas (5), bronchopneumonia (1), culture-negative acute pneumonia (1), respiratory bronchiolitis (1) and resolving infection (1). Diffuse alveolar damage cases involved non-specific lung injury which could be caused by drugs or non-specific infections and included organising pneumonia (6), non-specific interstitial pneumonitis (1), and non-specific lung injury (2).

Abbreviations: AF, antifungal; GVHD, graft versus host disease; PE, pulmonary embolism.

Histology samples were considered adequate in 22 cases (58%) and this was significantly different between the groups. All cases in groups A and B but only 8 cases (33%) in group C were considered adequate (P<0.001). The reasons for inadequate sample was not related to the biopsy method used (P= 0.369).

3.9.3 Adverse events

Biopsy was well tolerated. All pulmonary cases had pneumothorax but none required treatment and all had chest drains inserted in theatre. The drain was removed within 48 hours in 90% of cases. Mild to moderate pleural effusion requiring no treatment was seen in 6 cases (16%). Pain (mild/moderate) was seen in 7 cases (18%). There was no mortality or long term complications. One case of VATS was converted to open procedure due to haemorrhage in theatre. There were no cases of post-operative haemorrhage requiring interventions.

3.99 Diagnostic performance of different tests

The clinical performance of the available diagnostic tools used in this study is shown in Table 28. Because GM and BDG are used for the diagnosis of IFD, the clinical utility of these tests is measured against proven IFD and probable IFD using the alternative test. BDG has better sensitivity than GM even for the diagnosis of IA. Serum, compared to whole blood PCR has better sensitivity and PPV.

Table 28: Overall performance of the different diagnostic tests for detection of IA (or IFD where indicated)

	Sensitivity % (n)	**Specificity % (n)**	**PPV (% (n)**	**NPV % (n)**
GM	54 (49/91)	71 (27/38)	82 (49/60)	39 (27/69)
BDG				
IFD	79 (66/84)	55 (17/31)	83 (66/80)	49 (17/35)
IA	78 (65/83)	53 (17/32)	83 (65/80)	49 (17/35)
PCR (blood)				
IFD	15 (9/60)	97 (138/143)	75 (9/12)	73 (138/189)
IA	14 (8/59)	97 (138/143)	62 (8/13)	73 (138/189)
PCR (serum)				
IFD & IA	35 (17/48)	96 (21/22)	94 (17/18)	40 (21/54)
LFD	9 (4/46)	98 (311/316)	44 (4/9)	88 (311/353)
Biopsy	35 (7/20)	-	-	-

4.0 Risk factors associated with IFD, overall survival and prognosis

4.0.1 Risk factors for IFD

The Cox proportional Hazards method was used to assess potential risk factors for IFD. The factors assessed in the univariate model are shown in Table 29. A multivariate model using forward stepwise likelihood ratio was then created using variables with P ≥0.1 from the univariate model. The variables included in this model were Karnofsky score, index treatment, bacteraemia, neutrophil count, neutropenia ≥10days, monocytopenia ≥10days, baseline CT scan, hepatic dysfunction, itraconazole v others as first line prophylaxis, and ambisome v others as first line treatment. In addition the underlying haematological diagnosis was added to the model. The model significantly predicted IFD (omnibus χ^2= 24.88, df = 4, p<0.001). The results are shown in Table 30. Karnofsky score <90, bacteraemia, monocytopenia>10 days, and baseline CT scan abnormalities were significant independent risk factors for IFD. In addition, there was a trend for GVHD among allograft patients (HR 2.45, 95% CI 0.97-6.24), p=0.059). Neither steroids nor the PAM score were significant in the univariate analysis for allograft patients (HR [95% CI] 1.07 [0.94-1.17] and 0.79 [0.43-1.44] respectively).

Table 29: Univariate Cox proportional hazards model for risk factors for IFD

Variable	Hazard Ratio (95% CI)	P
Age	0.99 (0.97-1.02)	0.786
Sex, male v female	1.26 (0.70-2.28)	0.446
Race, white v others	1.12 (0.94-1.34)	0.203
Weight	1.00 (0.99-1.02)	0.632
Karnofsky score, <90 v ≥90	1.91 (1.04-3.51)	0.037
CMV serostatus, neg v pos	0.61 (0.26-1.45)	0.266
Diagnosis, MDS/AML v others	1.50 (0.81-2.78)	0.201
Index treatment, Others v autographs	5.70 (1.37-23.69)	0.017
Previous chemo/IST or HSCT, yes v no	1.30 (0.65-2.58)	0.454
Bacteraemia anytime, yes v no	2.55 (1.36-4.78)	0.003
Viral reactivation/infection, yes v no	1.30 (0.65-2.59)	0.460
Neutrophil count	0.71 (0.53-0.95)	0.020
Neutropenia >10d	2.30 (1.06-4.96)	0.034
Monocytopenia >10d	3.34 (1.79-6.25))	<0.001
Lymphopenia >10d	1.03 (0.47-2.22)	0.950
Baseline CT, abnormal v normal	2.34 (1.17-4.68)	0.016
Renal impairment, Creatinine >240μmol/l	0.87 (0.27-2.82)	0.817
Hepatic dysfunction	0.44 (0.24-0.80)	0.008
Itraconazole v others as first line prophylaxis	0.46 (0.24-0.86)	0.015
Ambisome v others as first line treatment	1.88 (0.9-3.92)	0.093

Table 30: Multivariate Cox proportional hazards model for significant risk factors for IFD

Variable	Hazard Ratio (95% CI)	P
Karnofsky score	2.11 (1.06-4.21)	0.034
Bacteraemia	2.48 (1.22-5.03)	0.013
Monocytopenia ≥10 days	2.64 (1.28-5.43)	0.009
Baseline CT scan	2.52 (1.21-5.26)	0.014

4.0.2 Overall survival

The mean follow-up period in this study was 556 days (range 12-730; 95% CI 519-593 days). The intense follow-up period where all study protocols were monitored was for a median of 194 days (range 12-647 days). The overall survival (OS) estimate at 3, 6, 12, and 24 months were 91%, 81%, 72% and 68% respectively for the whole cohort. Excluding non-IA cases (i.e. IA cohort) the OS was 92%, 83%, 76%, and 70% at 3, 6, 12, and 24 months respectively. Figure 46 shows the KM estimates of OS for both the whole cohort and the IA cohort. Overall survival was significantly lower in patients with proven/probable IFD compared to those with no evidence of IFD while patients with possible IFD and not classified group had an intermediate survival outcome (Figure 47). The survival estimates for the IA cohort was similar to the whole cohort.

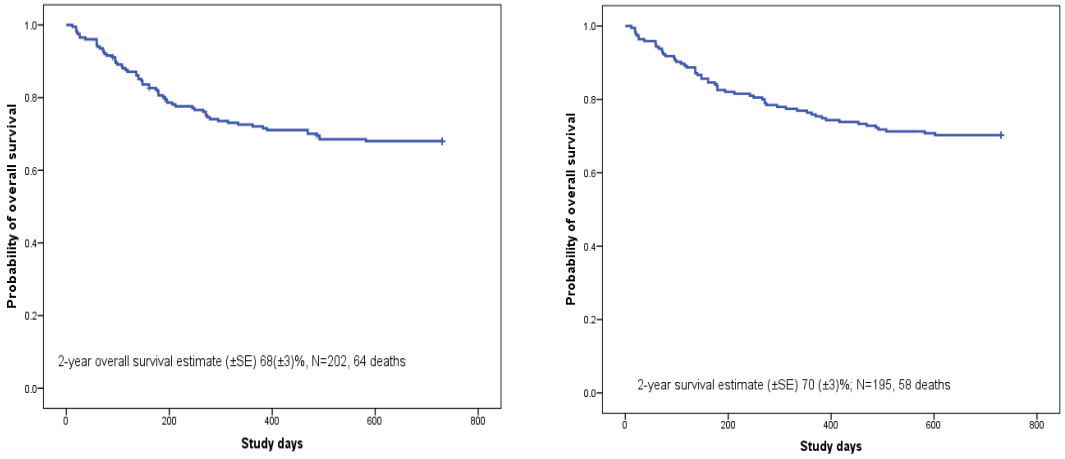


Figure 46: Overal survival analysis by Kaplan-Meier method of the whole cohort (left) and IA cohort cases (right).

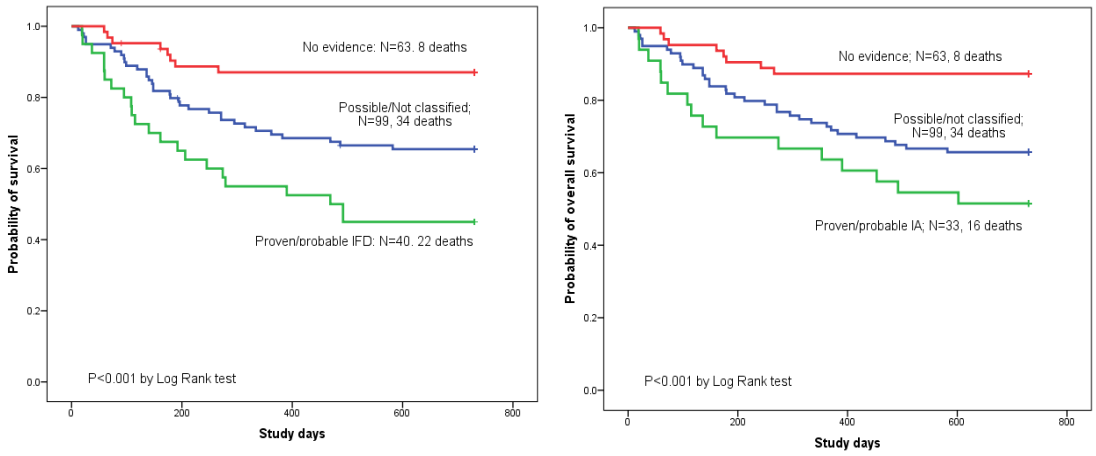


Figure 47: Kaplan-Meier analysis of overal survival for patients with proven/probable IFD vs others for the whole cohort (left) and for the IA cohort (right) . The 2-year survival estimates (95% CI) were 45 (29-61)%, 66 (56-76)%, and 87 (77-97)%, for proven/probable, possible/not classified and no evidence of IFD respectively.The corresponding estimates for the IA cohort (N=195) were 52 (34-70)%, 66 (56-76)%, and 87 (79-95)% for proven/probable IA, possible IA/not classified, and no evidence respectively.

4.0.3 Causes of mortality

There were 64 deaths in this cohort giving all-cause mortality rate of 32% at the end of the study period/follow-up. The causes of death are shown in Table 31. Relapse or progression of underlying haematological malignancy was the commonest cause of death (59%) while IFD accounted for 20% of deaths. Among the 40 patients who developed proven/probable IFD during the study period 18 (45%) were alive and 22 (55%) died at a median 296 days (95% CI 199-488) from the EORTC classification (Figure 48). The causes of death were relapse of haematological disease (6), IFD (13; proven *A. fumigatus*/mould NOS n=4, *Fusarium spp.* n=1, *C. glabrata* n=1, probable IA n=7)), GvHD (1), stroke (1) and PTLD (1). Therefore, the attributable mortality for IFD was 59% (13/22). However, 5 of the 38 deaths from relapse had active IFD at the time of death. Therefore, it could be argued that the true attributable IFD mortality was 82% (18/22). The median time between IFD diagnosis and death was 27 days for patients who died of IFD and 120 days for those who died of other causes. For patients from the IA cohort the median time between diagnosis and death was 25 days and 249 days for those who died from IA and those who died from other causes respectively.

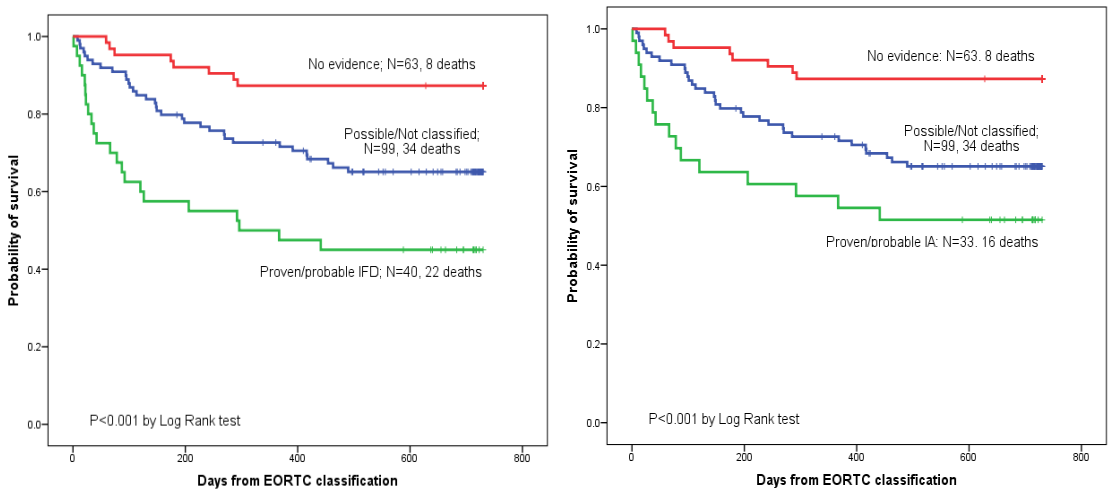


Figure 48: Survival estimates from EORTC classification to death/censor for the whole cohort (left) and the IA cohort (right). The median survival was only reached for the proven/probable IFD was 296 (95% CI 199-488) days.

Table 31: Causes of death

Cause of death, N=64	**N (%)**
Relapse/Progressive disease	38 (59.4)
IFD	13 (20.3)
MOF/sepsis other than IFD	5 (7.8)
Stroke	2 (3.1)
GVHD	2 (3.1)
Others*	4 (6.3)

*others included CNS toxoplasmosis post allograft (1), PTLD (1), accidental death (1), pulmonary haemorrhage (1)

4.0.4 Prognostic factors

Risk factors for death were analysed in a univariate Cox proportional Hazards model (Table 32). Factors with $p \leq 0.1$ were then used to construct a multivariate model. A total of 202 cases were analysed and the model significantly predicted death (Omnibus χ^2=77.25, df = 5, p<0.001). The underlying haematological diagnosis, bacteraemia anytime during the study period, ITU admission, hepatic dysfunction, and the index treatment received prior to the EORTC/MSG classification were the five independent prognostic factors that were significantly associated with death in the whole cohort (Table 33). A second multivariate Cox proportional Hazards model (Omnibus χ^2 = 87.56, df = 4, p<0.001) was created for patients with proven/probable IFD compared to no evidence of IFD. For this model 103 patients were included as possible and not classified patients were excluded in order to fully assess the potential role of IFD as a risk factor for mortality (Table 34). The survival plots for the IFD v no IFD, ITU admission status, and renal failure are shown in Figure 49 and the other independent prognostic factors from the multivariate model of the whole cohort in Figure 50.

Table 32: Univariate Cox proportional hazards model for prognostic factors for (N=202)

Variable	Hazard Ratio (95% CI)	P
Age	1.00 (0.98-1.02)	0.962
Sex, male v female	1.32 (0.79-2.21)	0.292
Race, white v others	1.62 (0.46-5.73)	0.457
Karnofsky score, <90 v ≥90	1.38 (0.81-2.36)	0.239
Diagnosis, AML/MDS v others	3.47 (2.06-5.86)	<0.001
Index treatment, Others v autographs	6.97 (2.79-17.37)	<0.001
Proven/probable IFD	2.58 (1.54-4.31)	<0.001
GM pos ≥2 times	2.30 (1.25-4.26)	0.007
Bacteraemia anytime, yes v no	2.48 (1.52-4.05)	<0.001
Viral reactivation/infection, yes v no	1.80 (0.98-3.31)	0.059
ITU admission	5.99 (3.18-11.24)	<0.001
Neutropenia >10d	1.30 (0.78-2.16)	0.317
Monocytopenia >10d	1.87 (1.14-3.06))	0.013
Lymphopenia >10d	1.65 (0.88-3.10)	0.117
No. of febrile episodes	1.12 (1.01-1.24)	0.025
Renal impairment Creatinine >240	2.91 (1.25-6.76)	0.013
Hepatic dysfunction	3.34 (2.02-5.52)	<0.001
Itraconazole v others as first line prophylaxis	0.80 (0.46-1.40)	0.437
Ambisome v others as first line treatment	0.71 (0.40-1.27)	0.251

Table 33: Multivariate Cox proportional hazards model for prognostic factors for the whole cohort

Variable	Hazard Ratio (95% CI)	P
ITU admission	3.05 (1.58- 5.88)	0.001
Index treatment, others v autografts	2.83 (1.00- 8.01)	0.050
Diagnosis MDS/AML v others	2.01 (1.13- 3.57)	0.018
Bacteraemia	1.90 (1.16- 3.14)	0.011
Hepatic dysfunction	1.88 (1.10- 3.18)	0.020

Table 34: Multivariate Cox proportional hazards model for prognostic factors for patients diagnosed with proven/probable IFD v no evidence of IFD*

Variable	Hazard Ratio (95% CI)	P
ITU admission	3.11 (1.03- 9.43)	0.045
Index treatment, others v autografts	4.51 (0.97- 21.03)	0.055
Proven/probable IFD	2.57 (1.03- 6.40)	0.042
Renal dysfunction	21.74 (4.59- 100.00)	0.011

*possible IFD and not classified cases were excluded from this analysis

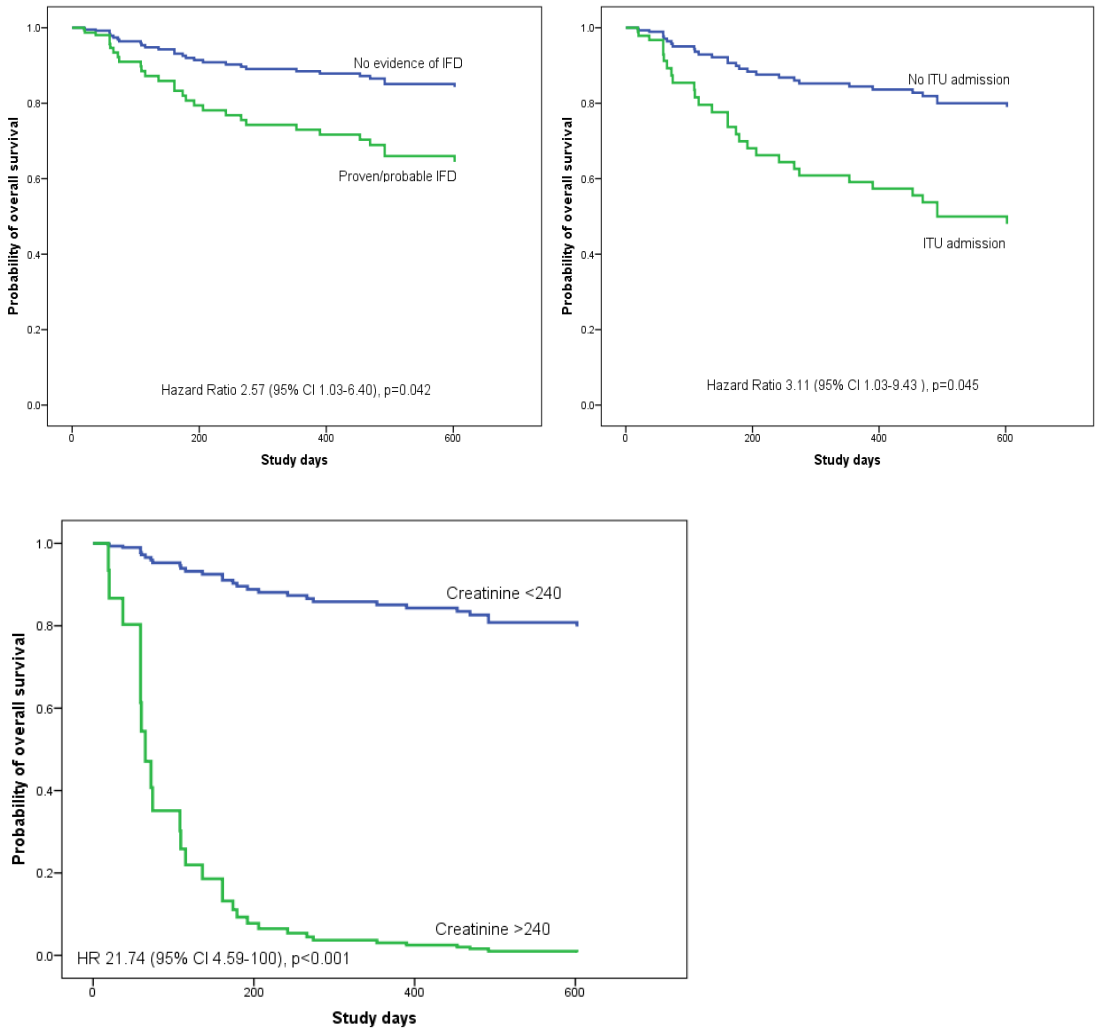


Figure 49: Survival plots for patients with IFD v no evidence of IFD (left), ITU admission v no admission (right), and renal failure (bottom) using the multivariate Cox proportional Hazard Ratio (N=103). The model included diagnosis, bacteraemia, viral infection, ITU admission, steroid use, monocytopenia, lymphopenia, renal and hepatic dysfunction, index treatment, no. of febrile episodes and proven/probable IFD.

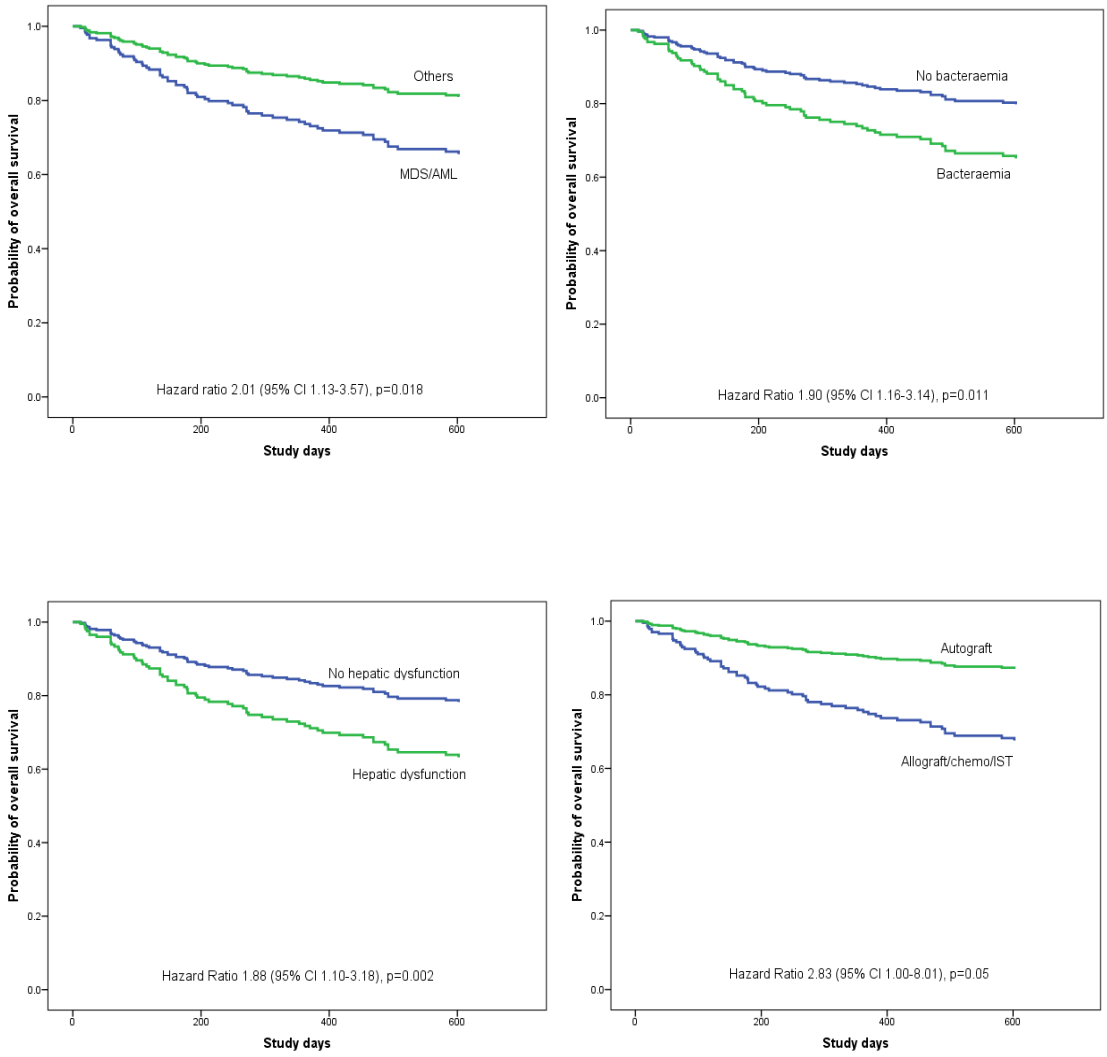


Figure 50: Survival plots of the whole cohort showing the effect of underlying diagnosis (top left), positive blood cultures (top right), hepatic dysfunction (bottom left), and type of treatment (bottom right) in a multivariate Cox proportional model (N=202).

Chapter 4 Radiology Results

4 Radiology results

4.1 Baseline scans

202 of the 203 patients recruited into the study had baseline CT chest scans at the time of recruitment. One patient did not have the scan due to cancellation of planned allogeneic HSCT and was discharged early. The median time from recruitment to scanning was 0 days (range 0-10 days) and 192 (95%) of the scans occurred within 3 days of recruitment. Table 35 shows the baseline scan primary signs.

Table 35: Baseline CT chest signs

CT findings	**No. (%) patients, N=202**
Normal	126 (62.4)
Nodules	16 (7.9)
Halo sign	6 (3.0)
Cavity	1 (0.5)
Ground glass opacification	8 (4.0)
Consolidation	16 (7.9)
Bronchiectasis	4 (2.0)
Micronodules	5 (2.5)
Tree-in-bud	4 (2.0)
Pleural abnormalities*	8 (4.0)
Other abnormalities†	8 (4.0)

*included pleural effusion (4), plaques (2), tag (1), thickening (1)

†included emphysema (3), sub-pleural reticulation (2), atelectasis (1), cysts (1), PE (1)

Seven of the 23 (30%) patients with nodules had a febrile episode within 24 hours of the scans and four of these seven patients subsequently fulfilled the criteria for probable IPA while 3 remained possible cases. One of these possible cases was rather unusual. He was a 55 year old man with CML and treated with imatinib and then dasatinib but with suboptimal response and was admitted for allogeneic HSCT. At admission his FBC were: Hb 10.6 g/dl, Neutrophils 7.16 $x10^9$/l and Platelets 346 $x10^9$/l. There were no previous CT scans performed at his local hospital and he had no neutropenic sepsis previous to admission. He was on itraconazole primary prophylaxis as per unit protocol. His baseline CT showed bilateral upper and lower lobe nodules with halo signs (Figure 51). Both GM and BDG were negative. Lung biopsy was planned but was not logistically possible during his conditioning chemotherapy.

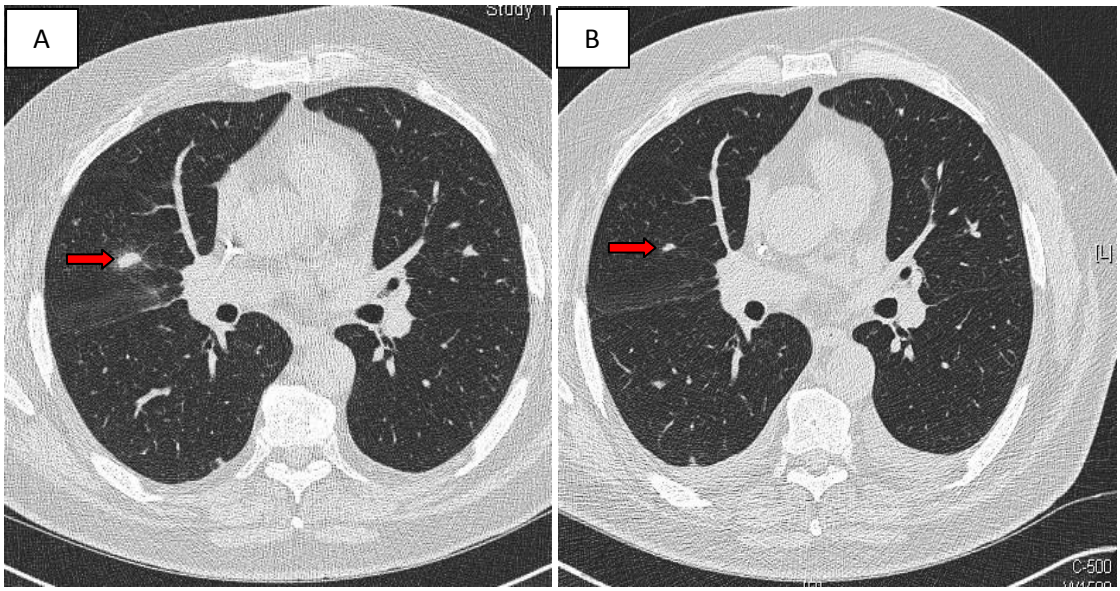


Figure 51: CT scan of JW 55y male with CML with normal neutrophil counts previously only treated with imatinib and dasatinib. He became febrile within 24-48h of starting conditioning chemotherapy. Baseline CT (A) showed nodules with halo sign (arrow). He was treated with Ambisome for 1 day and then changed to caspofungin

for 16 days and then voriconazole for 52 days. Follow up CT after weeks (B) shows some partial resolution.

4.1.1 Do the results of the baseline scan influence the incidence of IPA?

126 patients who had normal baseline CT scans were compared to 72 patients with baseline abnormalities (Figure 52 & Table 36). Four patients who had baseline abnormalities (nodules) and became febrile within 24h of the scan were excluded from this analysis (See section 4.1.0). Of the 126 patients with normal scans 62 (49.2%) had no evidence of IFD, 37 (29.4%) were not classified, 10 (7.9%) were possible IFD, 15 (11.9%) were probable IFD, 1 (0.8%) had *A. fumigatus*, and 1 (0.8%) had *Candida guilliermondii.* Therefore, the incidence of proven/probable IFD and IA among patients with normal baseline scan was 13.5% and 12.7% respectively. Of the 72 patients with baseline abnormalities 19 (26.4%) had EORTC recognised signs and 53 (73.6%) had non-EORTC signs. The EORTC signs were nodules (12), nodules with halo sign (6), and cavity (1). The patients' final classification was probable IFD (6), proven mould NOS (3), possible IFD (8), and alternative diagnosis on biopsy (2; Hodgkin lymphoma and Sclerosing haemangioma). Therefore, the cumulative incidence of proven/probable IFD in this group was 47.4%. The final EORTC/MSG classification of patients with non-EORTC signs were *A fumigatus* (1), proven mould NOS (1), *Candida glabrata* (1), *Fusarium* spp (1), probable IFD (7), possible IFD (5), and not classified (37). The incidence of IFD and IPA in this group was therefore 20.8% and 17.0% respectively.

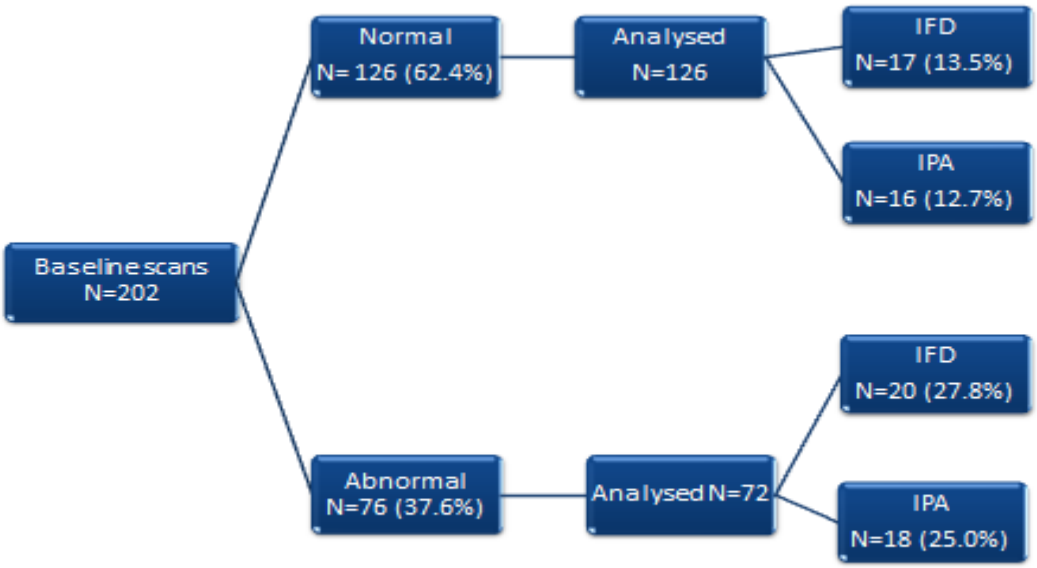


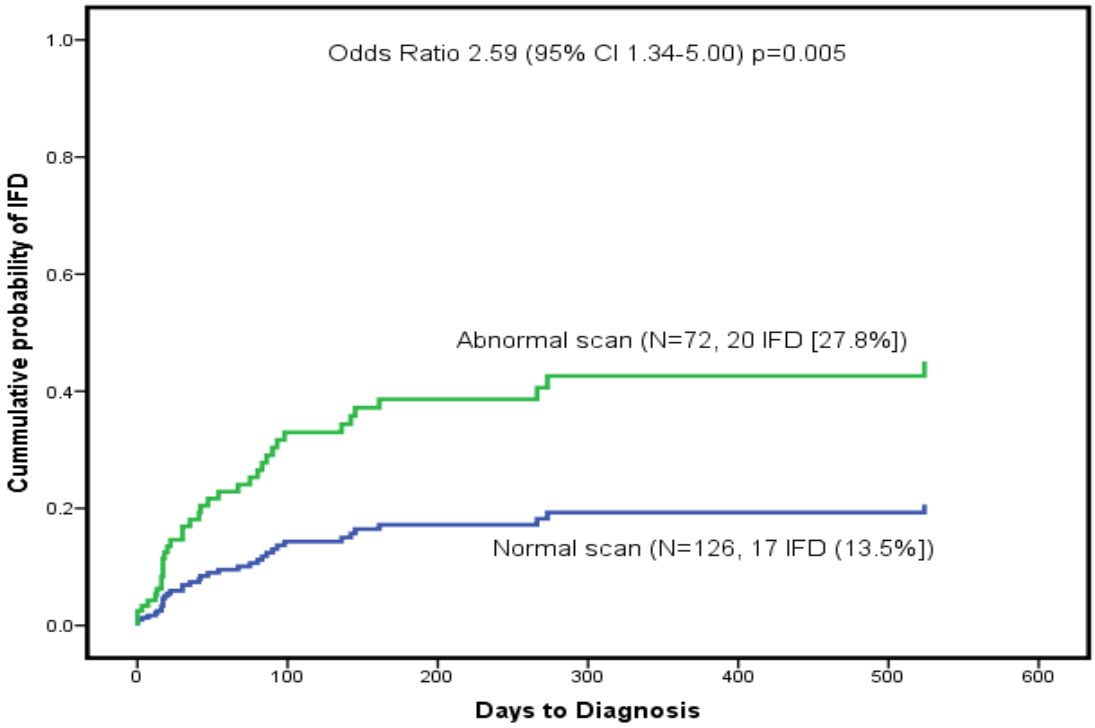
Figure 52: Outline of the baseline CT scan results

Table 36: Demographic and clinical characteristics of patients according to baseline pulmonary CT scan results

	Normal CT N=126	**Abnormal CT N=72**	**P-value**
Lesions	none	EORTC signs 19 (26.4%)* Non-EORTC signs 53 (73.6%)	-
Age, mean (95% CI), years	51.3 (49.1-53.4)	51.4 (48.1-54.7)	0.940
Males N (%)	79 (62.7)	42 (58.3)	0.544
Karnofsky score, mean (95% CI)	87 (86-89)	85 (82-87)	0.177
Pre-treatment, n (%)	108 (85.7)	61 (84.7)	0.849
Diagnosis			0.081
MDS	14 (11.1)	15 (20.8)	
AML	29 (23.0)	25 (34.7)	
NHL	19 (15.1)	9 (12.5)	
Myeloma	37 (29.4)	9 (12.5)	
Aplastic anaemia	13 (10.3)	6 (8.3)	
ALL	3 (2.4)	3 (4.2)	
Hodgkin lymphoma	4 (3.2)	3 (4.2)	
Others	7 (5.6)	2 (2.8)	
Treatment			0.005
Allogeneic HSCT	50 (39.7)	33 (45.8)	
Autologous HSCT	51 (40.5)	13 (18.1)	
Chemotherapy	18 (14.3)	21 (29.2)	
IST	7 (5.6)	5 (6.9)	
Baseline mean (95% CI)			
Neutrophil $x10^9$/l	3.5 (2.5-4.4)	2.1 (1.5-2.8)	0.050
Lymphocytes $x10^9$/l	1.0 (0.9-1.2)	1.1 (0.9-1.4)	0.533
Monocytes $x10^9$/l	0.5 (0.3-0.6)	0.3 (0.2-0.4)	0.173
Leucopenia, n (%)			
Neutropenia anytime	117 (93)	67 (93)	0.958
Neutropenia >10 d	70 (56)	50 (69)	0.054
Monocytopenia anytime	107 (85)	52 (72)	0.031
Monocytopenia >10 d	42 (33)	25 (35)	0.843
CRP, median (IQR) mg/l	5 (<5-19)	15 (<5-60)	NS
Time to first febrile episode, median (IQR),d	6 (0-9)	5 (2-18)	NS
Time to IPA, median (IQR),d	37 (15-68)	31 (7-85)	NS
IPA N (%)	16 (12.7)	18 (25.0)	0.002

*EORTC signs included nodules (12), halo sign (6), cavity (1) while non-EORTC signs were consolidation (16), ground glass opacification (8), pleural diseases (8; effusion [4], plaques [2], thickening [1], tag [1]), bronchiectasis (4), micronodules (5), tree-in-bud (4) emphysema (3), sub-pleural reticulation (2), atelectasis (1), cysts (1), and PE (1)

The cumulative incidences of proven/probable IFD and IPA in patients with normal and abnormal baseline CT scans were compared by Cox proportional hazard model. The model significantly predicted IFD (Omnibus $\chi^2 = 8.56$, df = 1, p = 0.003) and IPA (Omnibus $\chi^2 = 7.41$, df = 1, p = 0.006). Patients with abnormal baseline scan had a significantly higher incidence of IFD and IPA (Figure 53). The significance remained when other confounders were adjusted for in a multivariate model (see Table 30 in section 3.9.1). A further sub-analysis was performed to assess the impact of EORTC and non-EORTC signs compared to normal scans on the cumulative incidence of IFD (Omnibus $\chi^2 = 14.09$, df = 2, p = 0.001) and IPA (Omnibus $\chi^2 = 15.50$, df = 2, $p < 0.001$). Abnormal baseline scan with EORTC signs significantly predicted the incidence of both IFD and IPA while non-EORTC signs were statistically significant for predicting IPA (OR = 2.70, 95% CI 1.07-6.85) and a trend for IFD (OR = 2.21, 95% CI 0.91-5.35) (Figure 54).



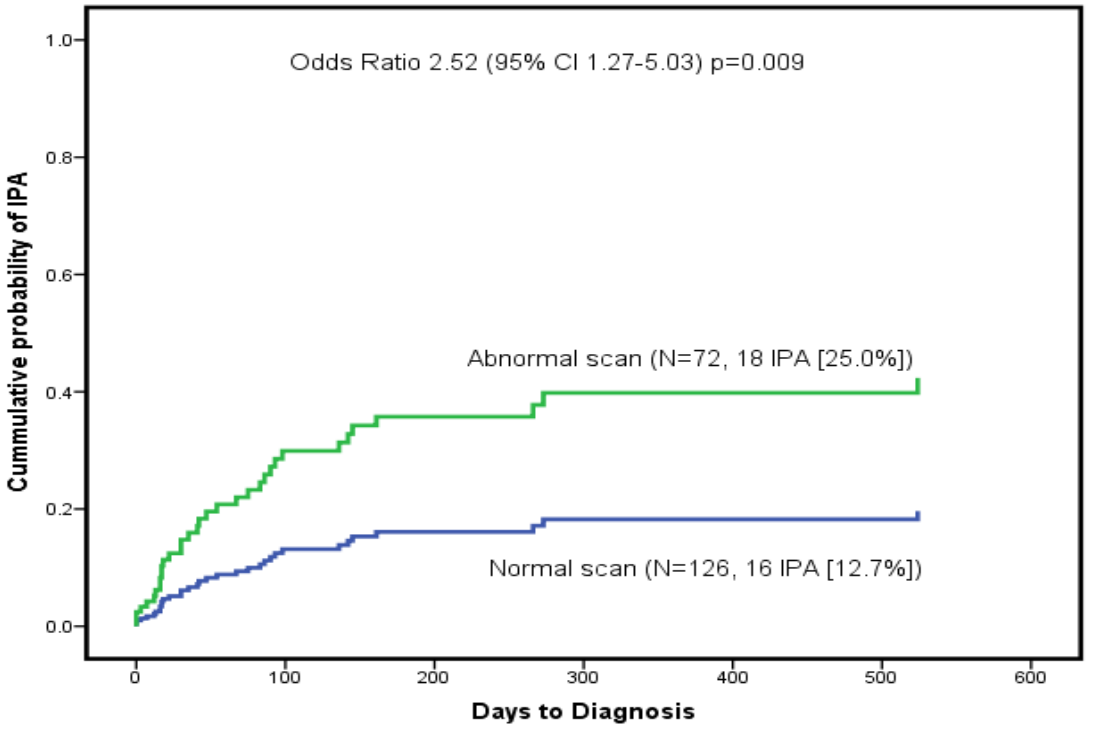
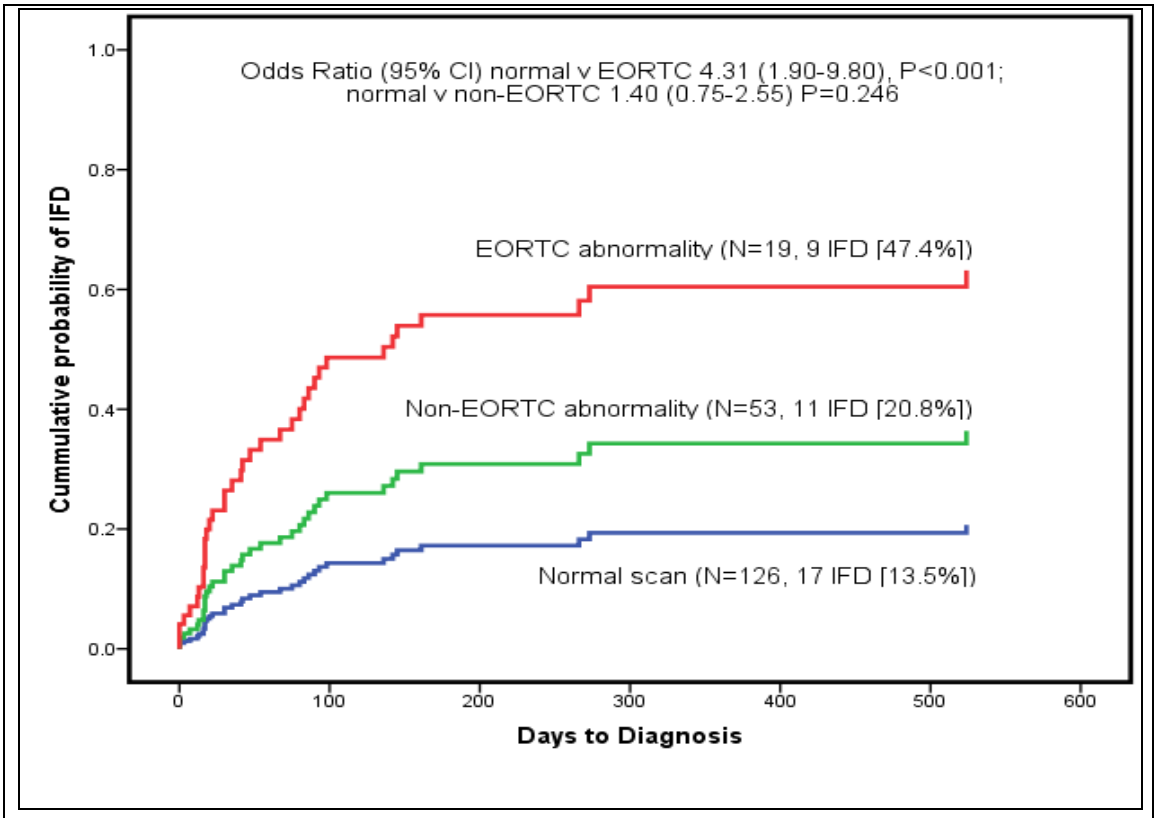


Figure 53: Cummulative incidence of IFD (top) and IPA (bottom) according to baseline CT scan results.



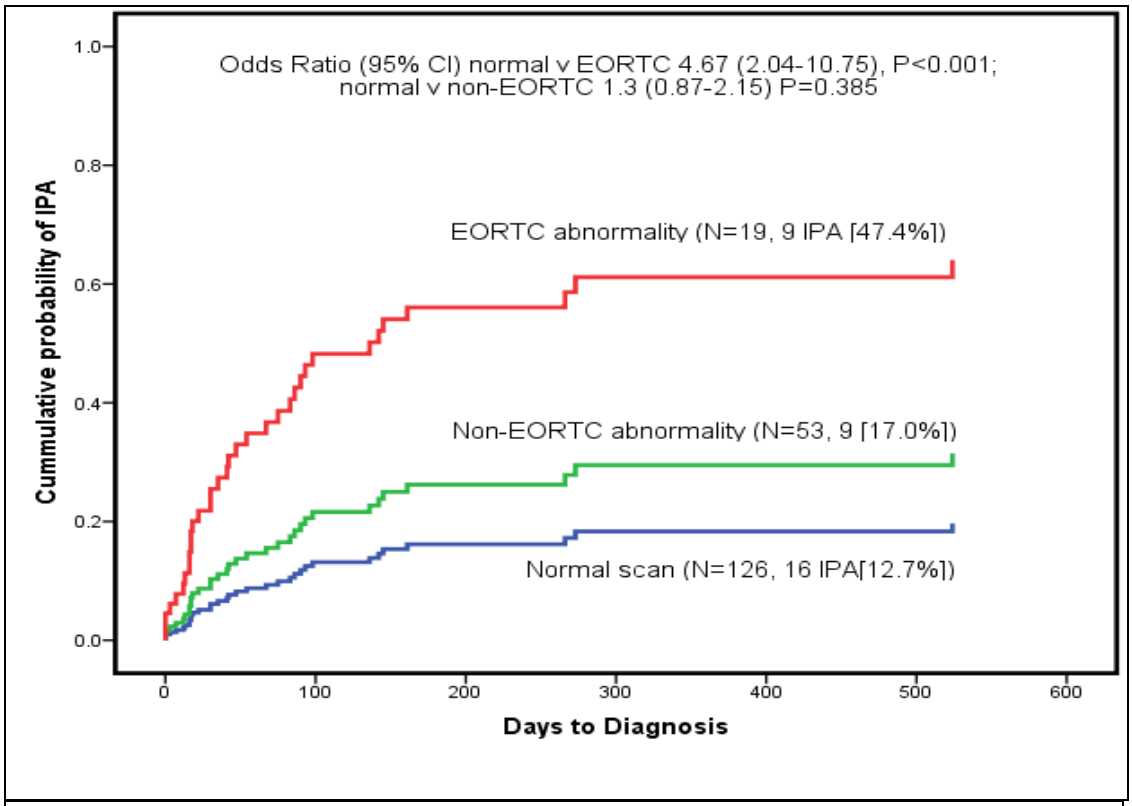


Figure 54: Cummulative incidence of IFD (top) and IPA (bottom) according to baseline CT scan results.

4.1.2 An unusual case of possible IPA

42 year old retailer diagnosed with AML FAB M4 with FLT3 ITD in May 2009. She was treated with 3 cycles of chemotherapy (DA, FLAG Ida x2) before being admitted for a matched unrelated donor allogeneic HSCT using the FB4C conditioning. She was in CR and had normal lung function tests and had no known previous history of IPA. She was recruited to the study in February 2010 and had baseline CT scan performed on the same day. This showed a nodule and consolidation in the left lower lobe (Figure 55). There was no supporting mycological evidence to upgrade to probable IPA. Her FBC was normal with Hb 11.1 g/dl, Neutrophils 2.55 $x10^9$/l and platelets of 153 $x10^9$/l. A VATS biopsy failed due to adherent pleura and CT guided biopsy was unsuccessful in obtaining adequate tissue. As per unit protocol she was on itraconazole prophylaxis. She had 3 neutropenic febrile episodes lasting for a total of 22 days. During these febrile episodes she responded to antibiotics adequately and grew *Micrococcus spp* from blood cultures taken from her Hickman line. She remained on itraconazole prophylaxis for a total of 153 days and received no additional antifungal treatment. A repeat scan 7 months later showed almost complete resolution of the opacity (Figure 55).

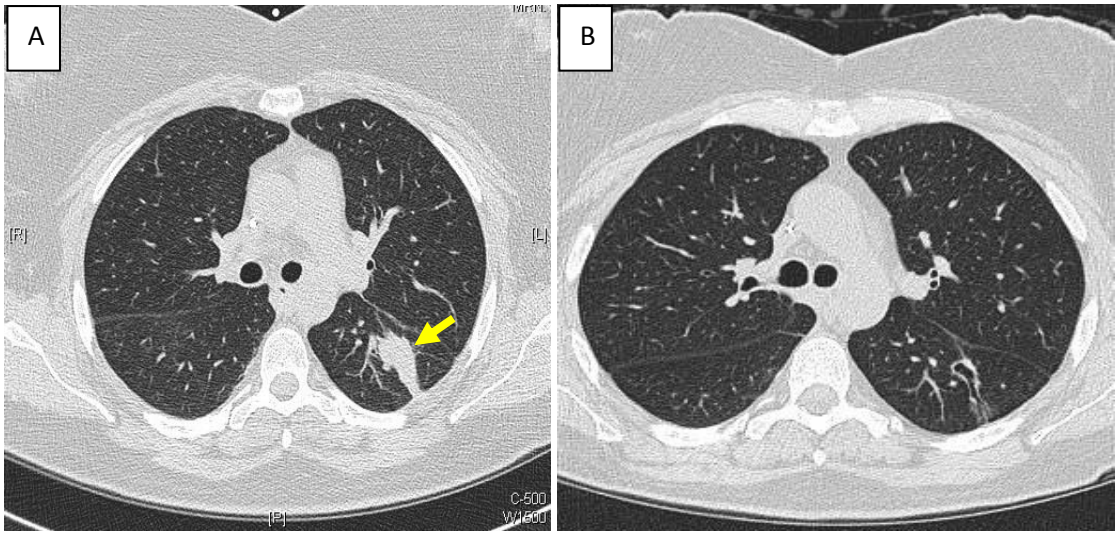


Figure 55: Baseline CT scan of the chest (A) showing a nodule in the left lower lobe (arrow). A repeat scan 7 months later showed resolution of the nodule (B) without antifungal therapy

4.2 Diagnostic scans

There were 151 diagnostic scans performed. Three were excluded: HRCT 1, incomplete lung exposure 2, leaving 148 evaluable scans among 108 patients (one scan n=84; 2 scans n=17; 3 scans n=3; 4 scans n=2; 5 scans n=1; 8 scans n=1). The median age was 52 years (range 19-73 years) and 54 (58%) were male. The diagnosis were MDS/AML (87), NHL (17), multiple myeloma (13), aplastic anaemia (11), ALL (8), Hodgkin lymphoma (7), MPD (4), CLL (1). The treatment they were receiving at the time of scan were allogeneic HSCT (n=89, 60%), autologous HSCT (n=27, 18%), and chemotherapy/IST (n=22, 22%). All the patients had neutropenic sepsis unresponsive to combination of broad-spectrum antibiotics for 72h or more and all received antifungal prophylaxis.

The CT signs were defined based on the recommendations of the Fleischner Society definitions [325]. IPA was diagnosed in 48 scans (proven n=7, probable n=41) and possible IPA was seen in 35 cases. Non-IPA cases included non-*Candida albicans* spp (3), *Fusarium* spp (1), not classified (38) and no evidence (24). The CT findings are shown in Table 37. Nodule/mass was the commonest type of lesion seen in patients with IPA, occurring in 46 (96%) cases.

Table 37: Diagnostic scan image findings

CT signs	**No. (%) of scans (N=148)**	**No. (%) of proven/probable IPA (N=48)**	**No. (%) of proven/probable/possible IPA (N=82)**
Nodule	77 (52)	44 (92)	75 (92)
Mass	12 (8)	8 (17)	11 (13)
Halo sign	41 (28)	21 (44)	39 (48)
Cavity	15 (10)	8 (17)	15 (18)
Ground glass opacity	68 (46)	27 (56)	49 (60)
Consolidation	70 (47)	25 (52)	48 (59)
Micronodule	31 (21)	12 (25)	21 (26)
Bronchiectasis	18 (12)	10 (21)	17 (21)
Bronchiectasis severity	19 (13)	9 (19)	17 (21)
Bronchial wall thickening	13 (9)	5 (10)	9 (11)
Tree-in-bud	5 (3)	1 (2)	2 (2)

4.2.1 Comparison of the CT signs between IPA and non-IPA patients

The CT findings seen in the diagnostic scans of patients are shown in Table 38. The presence of a nodule was the most frequent feature and highest odds ratio at 308 (95% CI 53.9-1759.5) for the diagnosis of IPA. The sensitivity and specificity of a nodule in proven/probable IPA was 92% and 97% respectively while its positive and negative predictive values were 96% and 94% respectively. Among the proven mould IFD the sensitivity and specificity of nodule was 100%. The halo sign was observed in 44% of this cohort with IPA. Its sensitivity, specificity, PPV, and NPV for the diagnosis of proven/probable IPA were 44%, 97%, 91% and 70% respectively. Two patients who had the halo sign were found to have Hodgkin lymphoma on open lung biopsy and *Acinetobacter baumannii* pneumonia/septicaemia on post mortem examination. Pulmonary mass was observed in 17% of cases and its sensitivity, specificity, PPV and NPV were 17%, 98%, 89% and 62% respectively. Cavitation was seen in 8 (17%) of proven/probable IPA. Its sensitivity, specificity, PPV, and NPV were 17%, 100%, 100% and 62% respectively.

The other radiological features which are not EORTC/MSG-recognised criteria assessed in this cohort included ground glass opacities (GGO), consolidation, micronodules, bronchiectasis, bronchial wall thickening, and tree-in-bud signs. GGO, micronodules and bronchiectasis were found in a statistically significantly higher proportion of IPA patients compared to non-IPA cases (Table 38). GGO was present in 27 (56%) of IPA cases. All the GGO cases showed nodules (26) or mass with halo (1). Consolidation was present in 25

(52%) of proven/probable IPA cases which also showed nodules (23), mass with halo (1) and cavity (1). All the 12 scans with micronodules also showed nodules. Tree-in-bud infiltrations were rare in this cohort.

Table 38: Comparison of the CT signs between proven/probable IPA and non-IPA cases

CT signs	No. (%) of cases with IPA (N=48)	No. (%) of cases without IPA (N=66)	P value
Nodule	44 (91.7)	2 (3.0)	<0.001
Mass	8 (16.7)	1 (1.5)	0.003
Halo sign	21 (43.8)	2 (3.0)	<0.001
Cavity	8 (16.7)	0 (0.0)	0.001
Ground glass opacity	27 (56.2)	18 (27.3)	0.002
Consolidation	25 (52.1)	23 (34.8)	0.066
Micronodule	12 (25.0)	7 (10.6)	0.042
Bronchiectasis	10 (20.8)	1 (1.5)	0.001
Bronchiectasis severity	9 (18.8)	2 (3.2)	0.007
Bronchial wall thickening	5 (10.4)	4 (6.1)	0.394
Tree-in-bud	1 (2.1)	3 (4.5)	0.481

4.2.2 Location and distribution of lesions

a) EORTC signs

The RUL and LLL were the commonest sites for nodules accounting for 47% of all nodules and the lingula had the lowest number (7% of nodules) (Figure 56). Of the 44 nodules among the proven/probable cases 33 (75%) had ≤3 nodules and 11 (25%) had >3 nodules. The distribution was bilateral in 21 (48%) cases

including all cases with >3 nodules. All the masses were unilateral but involved more than 1 lobe in 2 cases. The distribution of the halo sign mirrored that of the nodules (Figure 56). Of the 21 IPA scans with a halo sign 11 (52%) were bilateral. Cavities were found predominantly in the RUL and LLL (90% of cavities) (Figure 57) and unilateral with only one bilateral case seen.

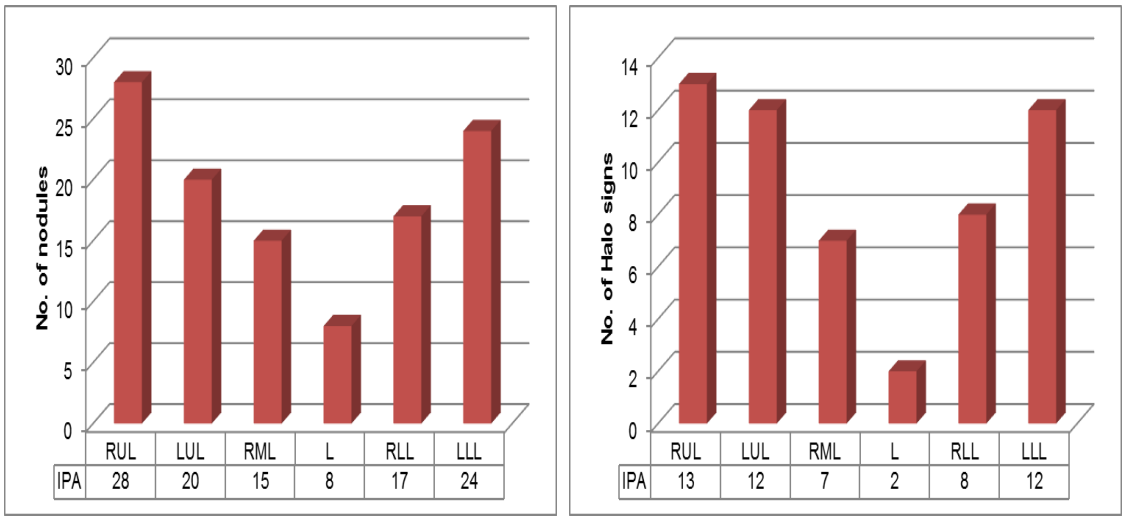


Figure 56: Distribution of nodules and Halo sign in the lungs among IPA patients.

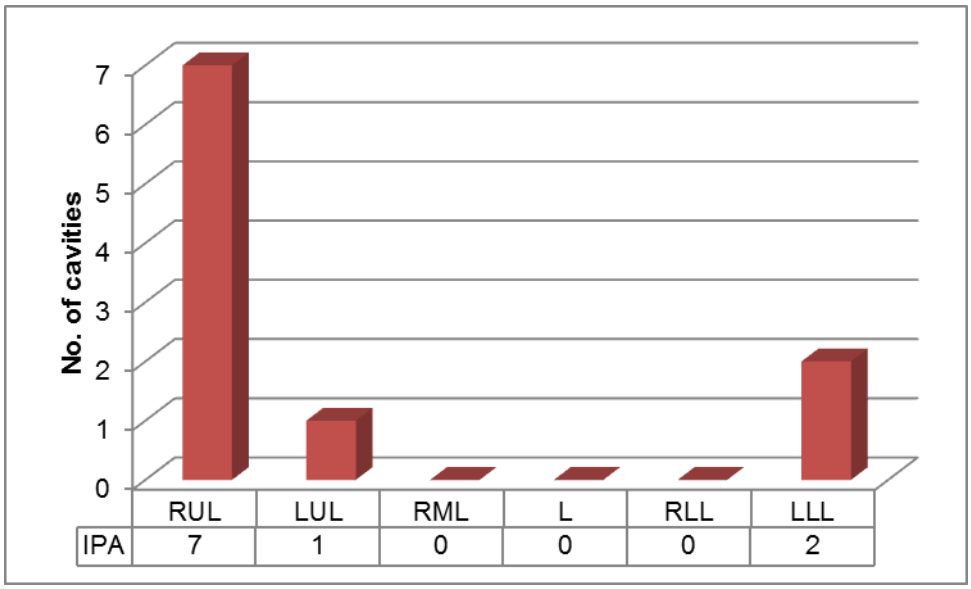


Figure 57: Distribution of cavities in IPA patients in different lobes of the lungs

b) Non-EORTC/MSG signs

The distribution of GGO was fairly uniform in the different lobes of the lungs (Figure 58) and bilateral in 17 (63%). Consolidation in IPA was bilateral in 14 (54%) and showed a predilection for the lower lobes where 60% occurred (Figure 58). Micronodules were bilateral in 25% of cases. All the bronchiectasis involved one broncho-pulmonary segment with none generalised and only one was bilateral. All the bronchiectasis as well as bronchial wall thickenings was trivial in severity.

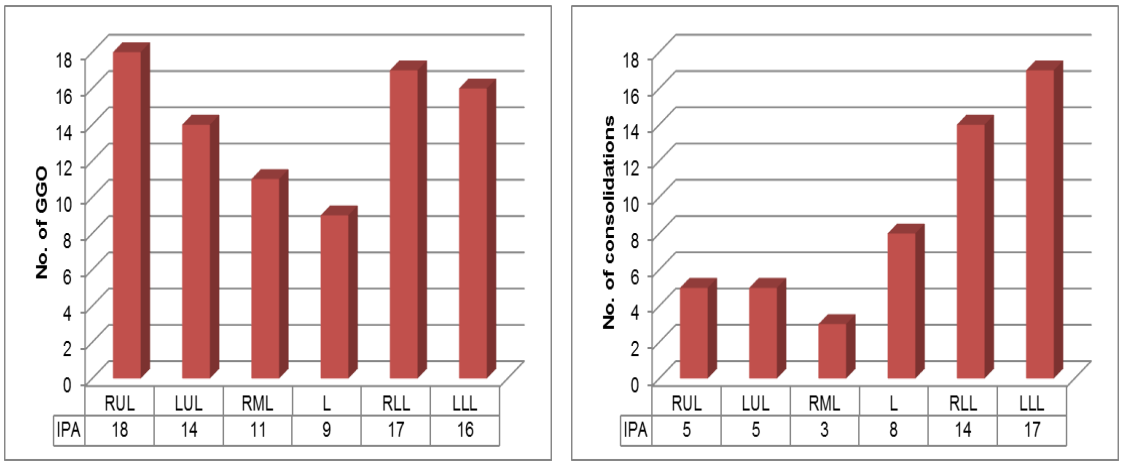


Figure 58: Distribution of GGO and consolidations in the lungs

4.2.3 Inter-observer variation for the diagnostic scans

Each lobe of the lungs was independently assessed by two consultant radiologists for both the EORTC/MSG signs and other radiological features and their agreement is shown in Table 39. Overall there was a statistically significant agreement between the consultants but the degree of agreement was higher with the EORTC/MSG signs compared to non-EORTC signs. Four cases would have been misclassified using a single radiologist in this cohort:

nodules in the RUL, RLL, LLL and lingula. In addition a halo sign would be missed in the RLL. Two of these possible cases were upgraded to probable IPA with the additional mycological evidence.

Table 39: Inter-observer agreement between the 2 radiologists in reporting CT scan of the chest

Lesion/location	Observed agreement (%)	Kappa Coefficient	P value
Nodule	83	0.702	<0.001
Mass	95	0.694	<0.001
Halo sign	88	0.692	<0.001
Cavity	97	0.796	<0.001
Bronchiectasis	93	0.657	<0.001
Bronchiectasis severity	90	0.553	<0.001
Bronchial wall thickening	93	0.414	<0.001
Tree-in-bud	97	0.489	<0.001

The inter-observer variation for continuous signs (percentage of consolidation, ground glass opacification, and micronodularity) was assessed by the Bland-Altman Plots in Figure 59. The degree of agreement between the 2 radiologists was moderately good. The mean difference (error) was random between the 2 observers.

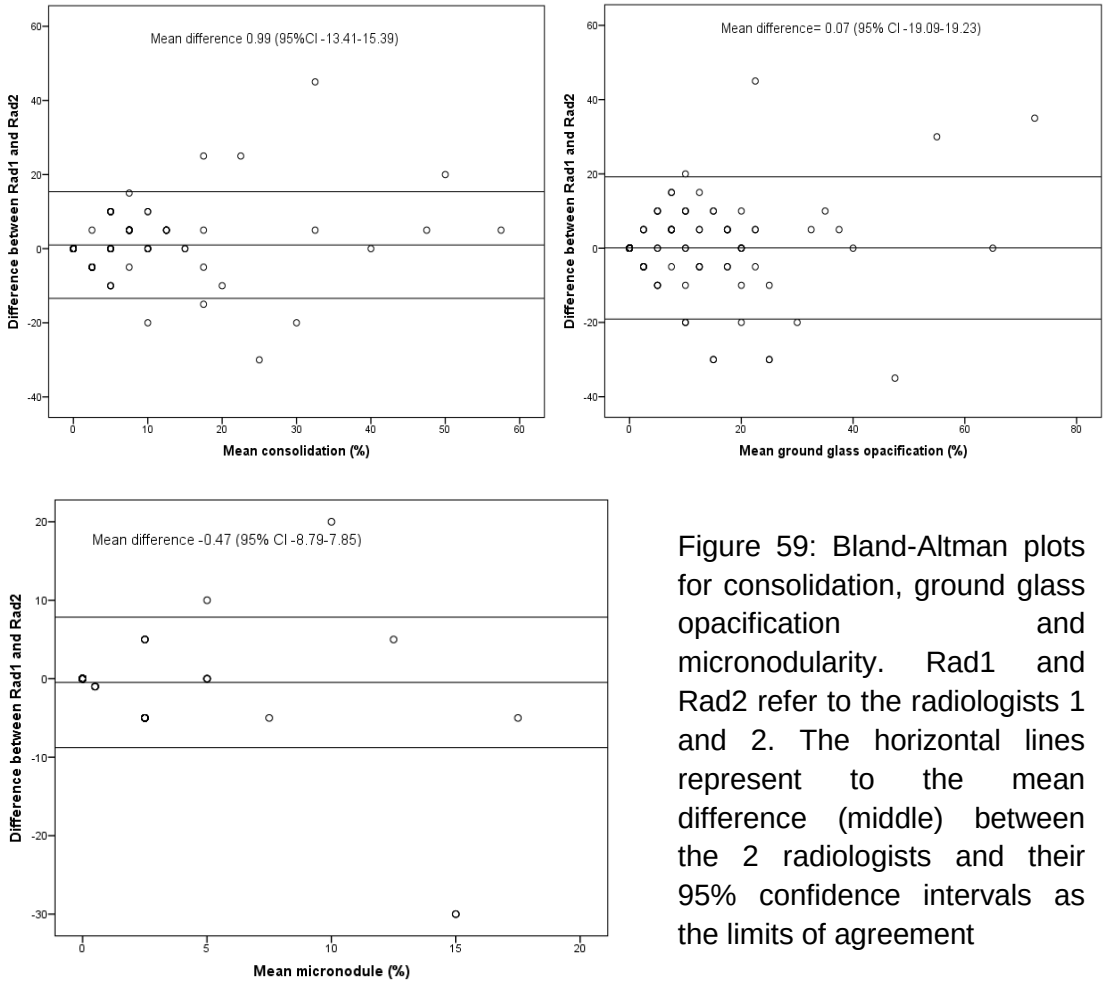


Figure 59: Bland-Altman plots for consolidation, ground glass opacification and micronodularity. Rad1 and Rad2 refer to the radiologists 1 and 2. The horizontal lines represent to the mean difference (middle) between the 2 radiologists and their 95% confidence intervals as the limits of agreement

4.2.4 Contrast enhancement

In order to assess the role of contrast enhancement in the diagnosis of IPA 27 patients (17M, 10F; MDS/AML 17, other diagnosis 7; allograft 16, autograft 4, chemotherapy/IST 7) had diagnostic contrast studies. The final EORTC/MSG classifications were proven mould (3), probable IFD (12), possible IFD (1), and not classified (6). The lesions in the not classified patients were consolidation (5) and consolidation and micronodules (1). The degree of enhancement over time was similar for both background lungs and the lesions of interests and demonstrates that the peak time of enhancement was 2-3 min from injection of contrast (Figure 60).

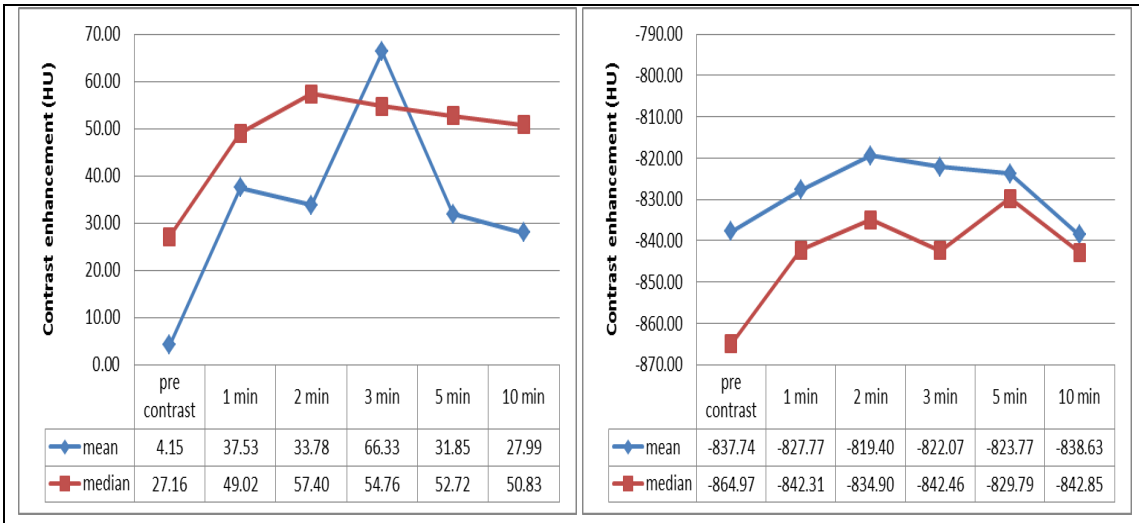


Figure 60: Degree of contrast enhancement in Housell Units at different time points. The enhancement pattern was similar for the lesions (left) and non affected background lungs (right)

Patients with proven/probable IPA were compared with those not classified (Table 40). Possible IPA was excluded due to uncertainty associated with this diagnosis and the risk of misclassification. No statistically significant difference was found between the 2 groups.

Table 40: Contrast enhancement in Hounsfield units (HU) between IPA and not classified patients

	Proven/probable IPA N=15	Not classified N=6	Mann-Whitney P-value
Median HU pre-contrast	27.2	21.7	0.519
Median HU at 1 min	39.9	50.8	0.424
Median HU at 2 min	57.4	45.8	1.000
Median HU at 3 min	50.9	53.8	0.677
Median HU at 5 min	52.7	59.4	0.519
Median HU at 10 min	50.8	47.6	0.850

4.3 Follow-up scans

A total of 76 follow-up scans were performed: 34 and 42 at follow-up 1 and 2, respectively. These scans were performed 10-14 days (follow-up 1) and 28-30 days (follow-up 2) post diagnostic scan to assess response to antifungal therapy. The EORTC/MSG response criteria were used to assess response. [321] Among the 48 proven/probable IPA cases 29 scans were performed, 10 could not be retrieved for scoring, 2 were post lung biopsy; the response rate among the 17 evaluable scans are shown in Table 41. The 19 scans that were not performed were due to early discharge (14), death (4) and patient refusal for additional scanning (1).

Table 41: Response rates among the IPA patients

	Follow-up 1	**Follow-up 2**
Complete response	1/17	2/13
Partial response	7/17	8/13
Stable disease	5/17	1/13
Progression	4/17	2/13

Successful response to therapy with complete or partial radiological response was achieved in 8 (47%) patients at 2 weeks compared to 10 (77%) at 4 weeks. Four patients had no follow-up 2 scans due to death (2) and early discharge (2). Among the 7 patients who achieved partial response (PR) at 2 weeks 3 remained in PR at 4 weeks, 1 progressed and subsequently died, 1 did not have a scan due to clinical instability, and 2 were discharged prior to follow-up 2 scan. One of the 2 patients who progressed at follow-up 2 died of IPA and the other achieved complete response on another scan done at 35 weeks upon neutrophil recovery.

Chapter 5 Cytokines Results

5 Cytokines results

5.1 Baseline cytokines

Baseline cytokine profiles were available for 172 (102 males, 70 females, median age 54, AML/MDS [69; 40%], aplastic anaemia [17; 10%], myeloma [43; 25%], NHL [29; 17%], others [14; 8%]) of the 203 patients. The other 31 patients had fever or chemotherapy administered before the cytokine samples were taken and were therefore excluded from this analysis. The final EORTC/MSG classifications were: proven *Aspergillus fumigatus* (2) mould NOS (1), *Fusarium* spp. (1), *Candida* spp. (4), probable IFD (21), possible IFD (14), not classified (67), and no evidence of IFD (62). The incidence of IFD and IA in this cohort was therefore 16.9% (29/172) and 14.4% (24/167) respectively. Proven/probable IFD and IA were considered true disease events while no evidence was considered as true negative. The possible and not classified cases were excluded from this analysis in order to avoid misclassification. The mean cytokine/chemokine concentrations of samples from patients with proven/probable IFD/IA were compared to those with no evidence of IFD using the Mann-Whitney method and the results are shown in Tables 38 and 39.

Five cytokines were found to be significantly different between IFD and no IFD cases. IL-15, IL-2R, MCP-1, and MIP-1α were significantly higher in IFD while IL-4 was significantly lower in patients who developed IFD compared to no IFD

(Table 43 and Figure 61). Patients who developed IA had significantly higher HGF, MCP1, and RANTES compared to those with no IA (Table 43 and Figure 62).

Table 42: Baseline cytokine profile comparing patients who developed proven/probable IFD to those with no evidence of IFD

Cytokine	**IFD N= 29**		**no IFD N=62**		**Mann-Whitney p-value**
	Median	IQR	Median	IQR	
EGF	65.1	21.6-208.4	82.4	29.5-218.1	0.285
EOTAXIN	74.7	31.0-150.0	63.4	37.8-100.6	0.740
FGF	14.6	5.5-27.4	13.0	9.2-22.5	0.692
GCSF	69.0	29.4-267.1	43.1	25.2-231.0	0.450
GMCSF	20.4	20.3-24.1	20.4	19.7-24.1	0.696
HGF	400.2	176.7-1346.1	179.2	67.9-829.8	0.071
IFN-α	27.7	20.3-48.1	23.6	19.1-34.3	0.259
IFN-γ	19.6	17.3-39.2	19.3	17.3-37.7	0.562
IL-10	8.4	6.0-10.9	9.4	5.8-13.1	0.762
IL-12	112.4	76.7-260.1	146.1	87.1-213.7	0.512
IL-13	27.4	25.7-49.4	25.7	20.7-35.6	0.223
IL-15	29.5	19.2-64.9	20.9	7.4-29.5	**0.035**
IL-17	16.9	10.6-16.9	16.9	11.8-17.1	0.243
IL-1β	16.9	9.8-19.2	14.1	9.8-16.9	0.303
IL-1RA	362.7	167.4-696.2	266.3	192.2-640.2	0.851
IL-2	6.7	4.8-13.7	6.4	5.0-9.9	0.746

IL-2R	616.7	322.3-1208.9	411.3	284.1-730.8	**0.039**
IL-4	9.0	4.0-36.1	33.8	9.0-51.6	**0.010**
IL-5	7.0	2.6-7.0	7.0	3.4-7.0	0.632
IL-6	7.9	3.4-30.7	7.9	4.4-15.3	0.645
IL-7	15.1	6.7-27.3	24.9	6.5-28.9	0.746
IL-8	86.9	51.6-470.2	64.2	27.3-284.5	0.193
IP-10	44.1	23.2-169.3	43.9	26.1-75.1	0.676
MCP-1	785.8	353.2-1421.8	333.5	144.9-620.8	**0.003**
MIG	85.9	43.2-168.5	85.9	52.4-182.2	0.925
MIP-1α	37.2	25.2-125.0	28.6	20.2-48.1	**0.049**
MIP-β	70.5	37.0-136.0	65.2	44.9-133.2	0.939
RANTES	3609.6	600.7-7622.6	6510.0	4958.2-8300.0	0.074
TNF-α	4.8	4.0-7.3	4.6	2.3-7.3	0.426
VEGF	15.9	3.7-22.0	22.0	3.6-29.8	0.453

Table 43: Baseline cytokine profile comparing patients who developed proven/probable IA to those with no evidence of IA

Cytokine	**IA N=24**		**no IA N=62**		**Mann-Whitney p-value**
	Median	IQR	Median	IQR	
EGF	69.7	29.7-275.5	80.7	29.5-218.1	0.591
EOTAXIN	72.8	33.1-166.3	64.9	37.8-100.6	0.701
FGF	14.6	11.9-61.8	13.7	9.2-22.5	0.423
GCSF	61.0	27.4-420.7	44.8	25.2-231.0	0.633
GMCSF	20.4	20.3-24.1	20.4	19.7-24.1	0.985
HGF	413.0	218.4-2045.8	179.7	67.9- 829.8	**0.041**
IFN-α	24.2	20.3-48.1	24.2	19.1-34.3	0.343

IFN-γ	19.3	10.8-38.9	19.3	17.3-37.7	0.989
IL-10	8.4	6.0-11.2	9.5	5.8-13.1	0.864
IL-12	124.2	82.6-260.1	145.2	87.1-213.7	0.824
IL-13	26.7	25.7-35.5	25.7	20.7-35.6	0.513
IL-15	24.2	19.2-58.5	21.1	7.4-29.5	0.130
IL-17	16.9	10.6-16.9	16.9	11.8-17.1	0.178
IL-1β	16.9	9.8-16.9	14.4	9.8-16.9	0.385
IL-1RA	433.0	199.5-779.0	266.3	192.2-640.2	0.494
IL-2	5.9	4.8-13.7	6.7	5.0-9.9	0.850
IL-2R	576.7	322.3-1104.5	412.0	284.1-730.8	0.091
IL-4	10.8	4.7-50.1	32.4	9.0-51.6	0.068
IL-5	7.0	2.6-7.0	7.0	3.4-7.0	0.993
IL-6	7.4	3.4-17.6	7.9	4.4-15.3	0.376
IL-7	25.2	8.4-27.3	24.8	6.5-28.9	0.959
IL-8	85.1	41.9-331.6	64.8	27.3-284.5	0.327
IP-10	43.3	23.2-149.4	43.7	26.1-75.1	0.926
MCP-1	673.5	353.2-1242.1	334.4	144.9-620.8	**0.013**
MIG	93.2	46.6-168.5	87.0	52.4-182.2	0.736
MIP-1α	35.3	25.2-125.0	29.0	20.2-48.1	0.079
MIP-β	70.5	41.2-136.0	66.7	44.9-133.2	0.882
RANTES	3609.6	600.7-6510.0	6510.0	4958.2-8300.0	**0.042**
TNF-α	4.0	2.8-7.3	4.6	2.3-7.3	0.801
VEGF	22.0	3.6-22.0	22.0	3.6-29.8	0.616

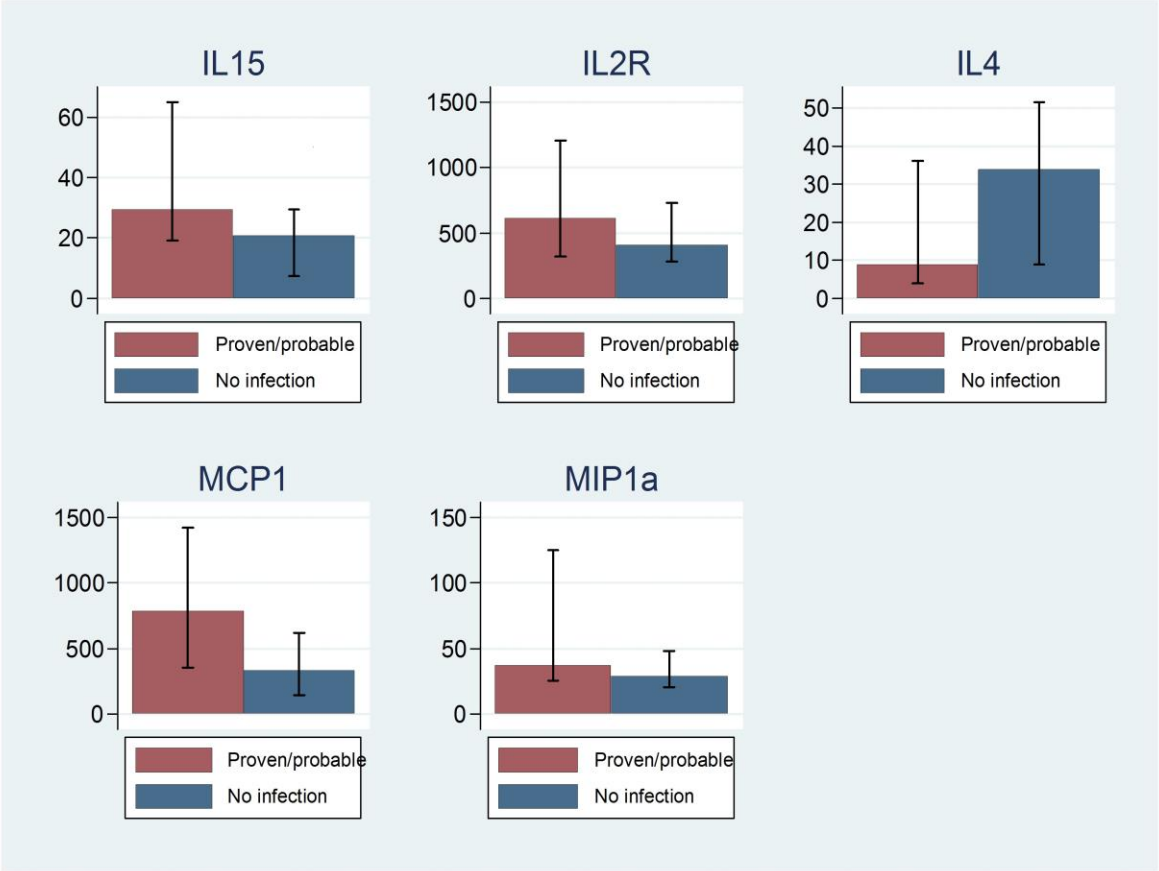


Figure 61: Median (with IQR) cytokine concentrations which were statistically significantly different by Mann-Whitney test between patients who developed IFD v no IFD

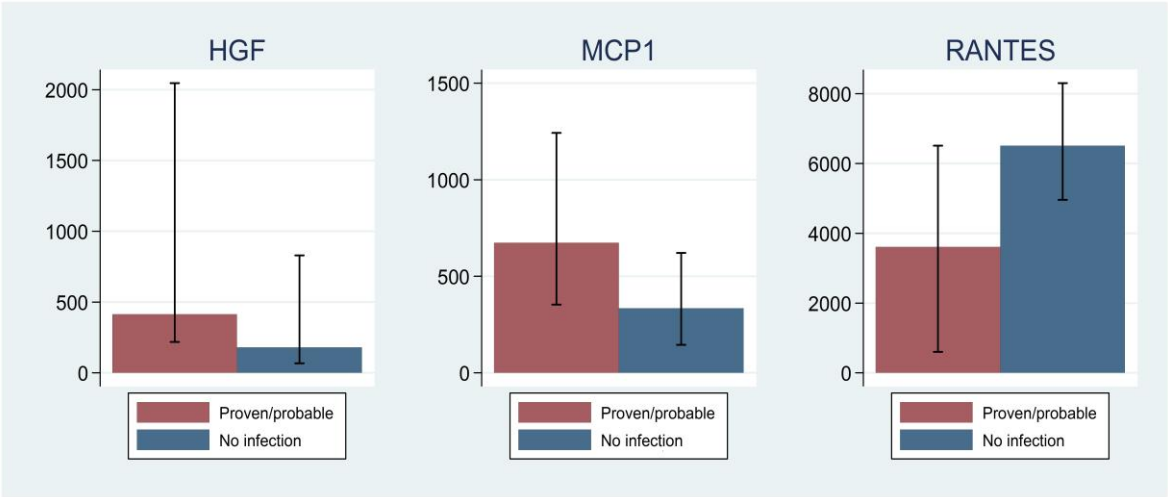


Figure 62: Median (with IQR) cytokine concentrations which were statistically significantly different by Mann-Whitney test between patients who developed IA v no IA

The risk of IFD and IA were assessed in a univariate Cox proportional hazard model for baseline cytokines that were statistically significantly different between IFD/IA cases and those who had no evidence of IFD/IA. A multivariate Forward Stepwise (Likelihood Ratio) model was then created and included IL15, IL-2R, IL-4, MCP, MIP-1α, diagnosis, index treatment, bacteraemia, monocytopenia>10 days, Karnofsky score, and baseline CT scan. The final model significantly predicted the incidence of IFD (Omnibus χ^2 = 42.32, d.f. = 3, p<0.001). The univariate model for HGF and IA (Omnibus χ^2 = 2.08, p = 0.149) and RANTES and IA (Omnibus χ^2 = 0.04, p = 0.949) did not fit well and were excluded from the final model. Therefore, no specific model was created for IA.

The multivariate model showed that high MCP-1, IL-2R, and bacteraemia were independent risk factors for IFD (Table 44 and Figure 63). The median MCP-1 and IL-2R concentrations were 419.0 pg/ml (IQR 150.2-840.6 pg/ml) and 443.8 pg/ml (IQR 297.0- 833.6 pg/ml) respectively. Among the 19 patients with MCP-1 above IQR, 13 (68.4%) developed IFD compared to 16 of the 72 patients (22.2%) with IL-2R ≤ IQR (Pearson χ^2 = 14.78, p<0.001). The Hazard or Odds ratio for IFD in patients with MCP-1 >IQR was 2.7 (95% CI 1.2-6.1). Of the 24 patients with IL-2R >IQR, 13 (54.2%) developed IFD compared to 16 of the 67 patients (23.9%) with serum concentrations ≤ IQR (Pearson χ^2 = 7.47, p = 0.006). The Hazard or Odds ratio for IFD in those with IL-2R >IQR was 2.3 (95% CI 1.1-5.1). Low IL-4

was a risk factor for IFD in the univariate model but this did not reach statistical significant in the multivariate model (Table 44).

Table 44: Cox regression model on baseline cytokines for the development of IFD

Cytokine concentration (pg/ml)	**Unadjusted**		**Adjusted**	
	Hazard Ratio (95% CI)	P-value	Hazard Ratio (95% CI)	P-value
MCP-1	4.6 (2.2-9.6)	<0.001	2.7 (1.2-6.1)	**0.016**
IL-2R	3.2 (1.5-6.7)	0.002	2.3 (1.1-5.1)	**0.037**
IL-4	3.5 (1.7-7.3)	0.001	2.5 (0.9-7.2)	0.090
MIP-1α	2.3 (1.1-4.8)	0.032	1.7 (0.7-4.2)	0.279
IL-15	2.8 (1.3-5.9)	0.008	1.2 (0.5-2.9)	0.759

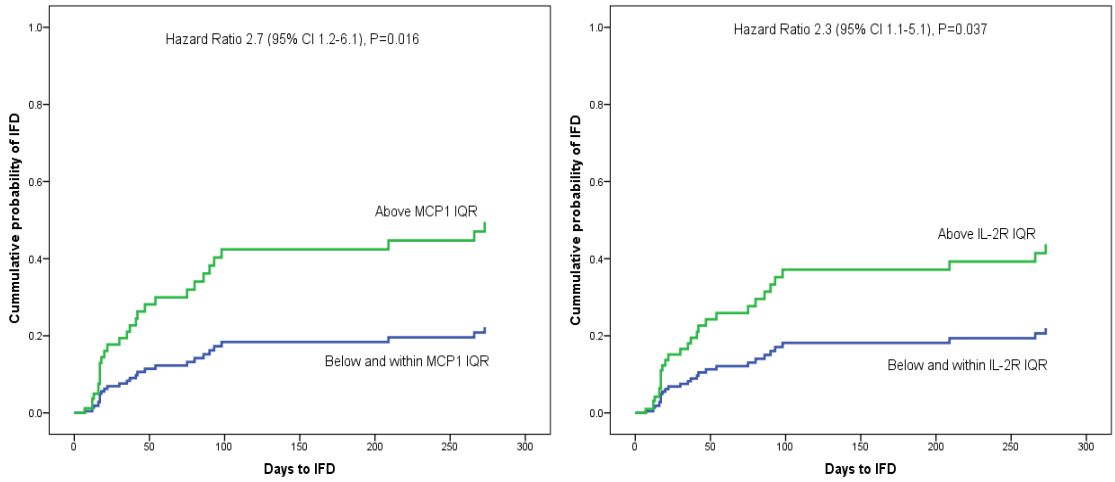


Figure 63: Cummulative probability (incidence) of IFD by multivariate Cox regression model 1-survival plot according to baseline MCP-1 (left) and IL-2R (right) serum concentrations.

Does the baseline cytokine concentration have any prognostic significance?

Baseline cytokine serum levels were assessed as potential prognostic factors using the Cox regression model. A univariate model was created for each of the 30 cytokines and the results are shown in Table 45. High IL-13 and IL-6 were associated with increased risk of death with risk/odds ratio of 1.8 (95% CI 1.0-3.4) and 2.4 (95% CI 1.3-4.3) respectively.

Table 45: Baseline cytokine levels as prognostic factors using univariate Cox regression model. In all cases cytokine levels within and below IQR were used as the indicator parameter

Cytokine	Hazard Ratio (95% CI)	p-value
EGF	1.0 (0.5-2.0)	0.989
EOTAXIN	1.0 (0.5-2.0)	0.924
FGF	0.5 (0.2-1.2)	0.126
GCSF	1.4 (0.7-2.6)	0.328
GMCSF	1.3 (0.7-2.6)	0.392
HGF	1.4 (0.6-2.8)	0.433
IFN-α	0.7 (0.3-1.5)	0.316
IFN-γ	1.3 (0.7-2.4)	0.466
IL-10	0.9 (0.4-1.7)	0.679
IL-12	1.0 (0.5-2.1)	0.913
IL-13	1.8 (1.0-3.4)	**0.048**
IL-15	1.6 (0.9-3.0)	0.125
IL-17	1.1 (0.5-2.2)	0.849
IL-1β	1.2 (0.6-2.3)	0.612
IL-1RA	1.3 (0.6-2.3)	0.530
IL-2	1.0 (0.5-2.0)	0.906
IL-2R	1.7 (0.9-3.1)	0.108
IL-4	0.7 (0.4-1.3)	0.256
IL-5	0.7 (0.4-1.4)	0.323
IL-6	2.4 (1.3-4.3)	**0.004**
IL-7	1.5 (0.8-2.8)	0.211
IL-8	1.1 (0.6-2.2)	0.714
IP-10	1.1 (0.6-2.2)	0.741
MCP-1	1.3 (0.7-2.5)	0.411
MIG	1.3 (0.7-2.4)	0.458

MIP-1α	1.1 (0.6-2.2)	0.713
MIP-β	1.1 (0.6-2.2)	0.680
RANTES	1.0 (0.4-2.9)	0.980
TNF-α	0.9 (0.5-1.6)	0.745
VEGF	1.1 (0.6-2.1)	0.792

A multivariate model was created which included IL-6, IL-13, IL-2R, ITU admission, hepatic dysfunction, bacteremia, underlying haematological diagnosis, and index haematological treatment (Omnibus χ^2 = 61.64, d.f. = 8, p<0.001). Using the forward stepwise (Likelihood Ratio) method the final model predicted death accurately (Omnibus χ^2 = 55.91, d.f. = 4, p<0.001). ITU admission, bacteremia, diagnosis, and index haematological treatment were independent prognostic factors. None of the cytokines were statistically significant factors.

5.2 Follow-up cytokines

A total of 412 serum follow-up samples from the 172 patients who had evaluable baseline serum concentrations were analysed. The median number of follow up cytokine samples per patient was 2 (range 2-15). Each patient was followed up from no evidence of IFD through to final diagnosis every 2 weeks during inpatient admission. Each of these inpatient admission episodes represents an at-risk period for IFD as these coincide with chemotherapy, IST, transplantation or its complications. Figure 64-67 shows the different cytokine panels at different time

points during the follow-up period. Overall no significant differences could be detected between patients with IFD v no IFD. However, IL-2R overall was consistently higher in IFD patients and appeared to increase with longer follow up samples (Figure 66).

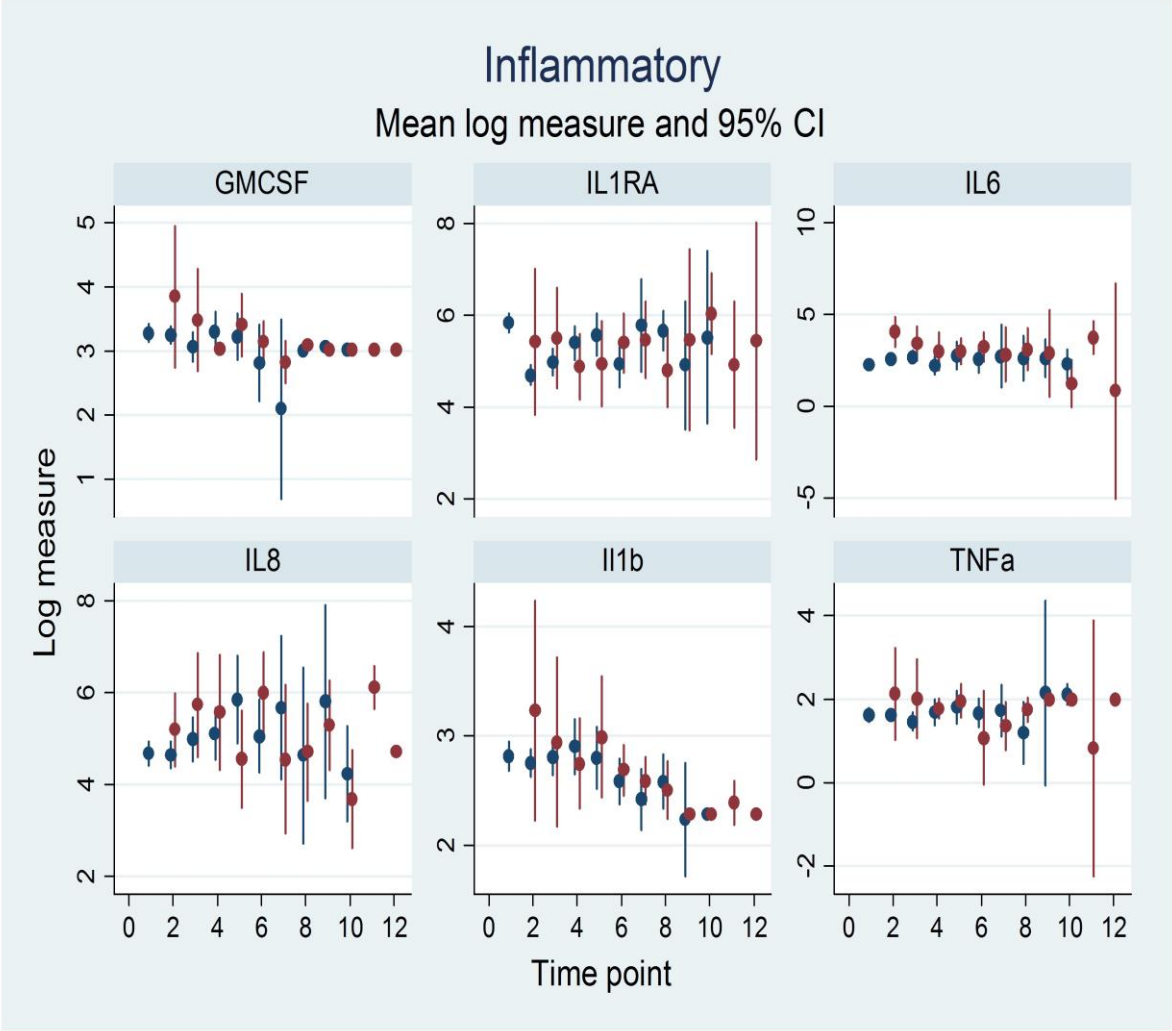


Figure 64: Inflammatory cytokine panel at follow-up for IFD (maroon) v no IFD (dark blue)

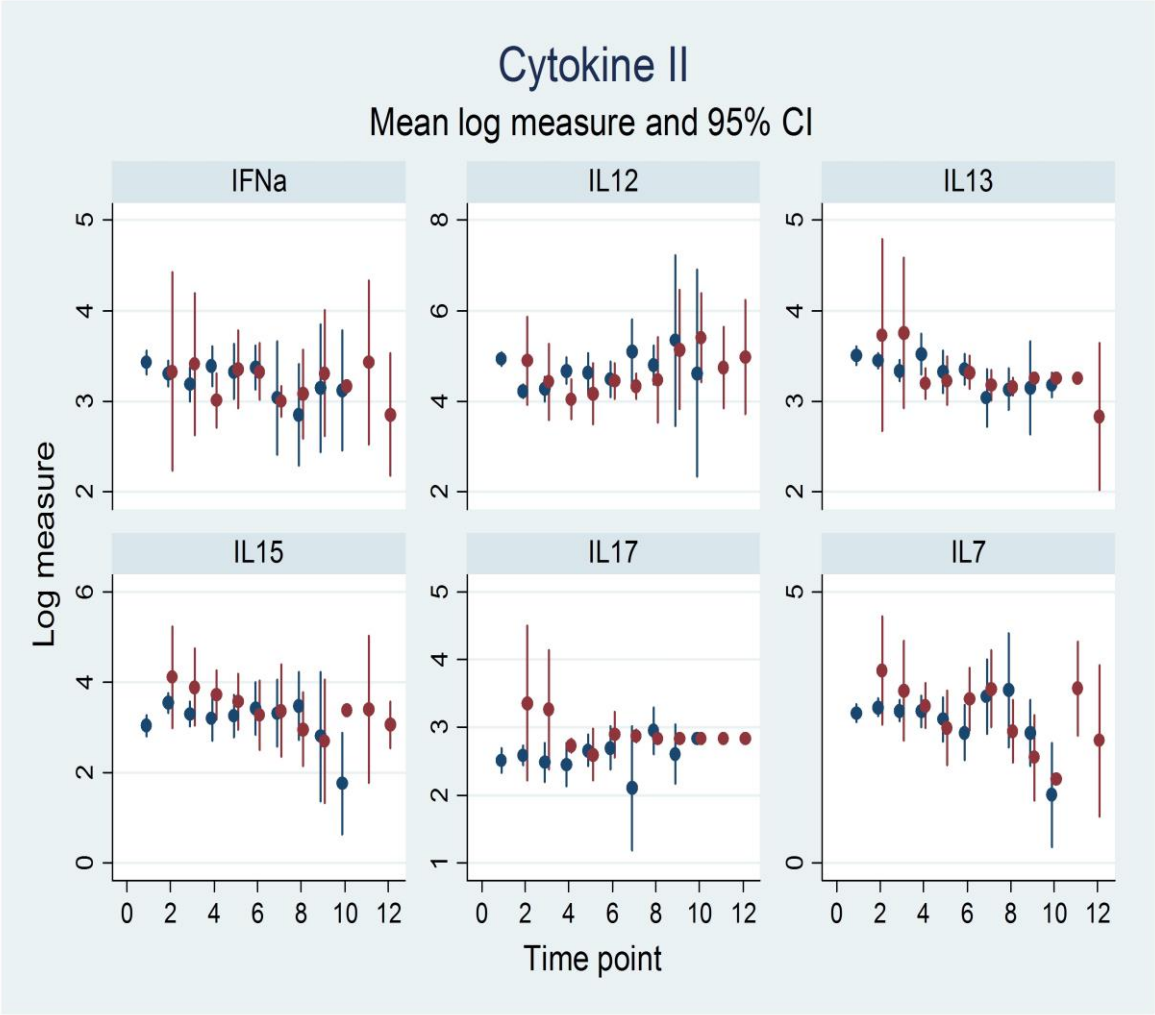


Figure 65: Inflammatory cytokine II panel at follow-up for IFD (maroon) v no IFD (dark blue)

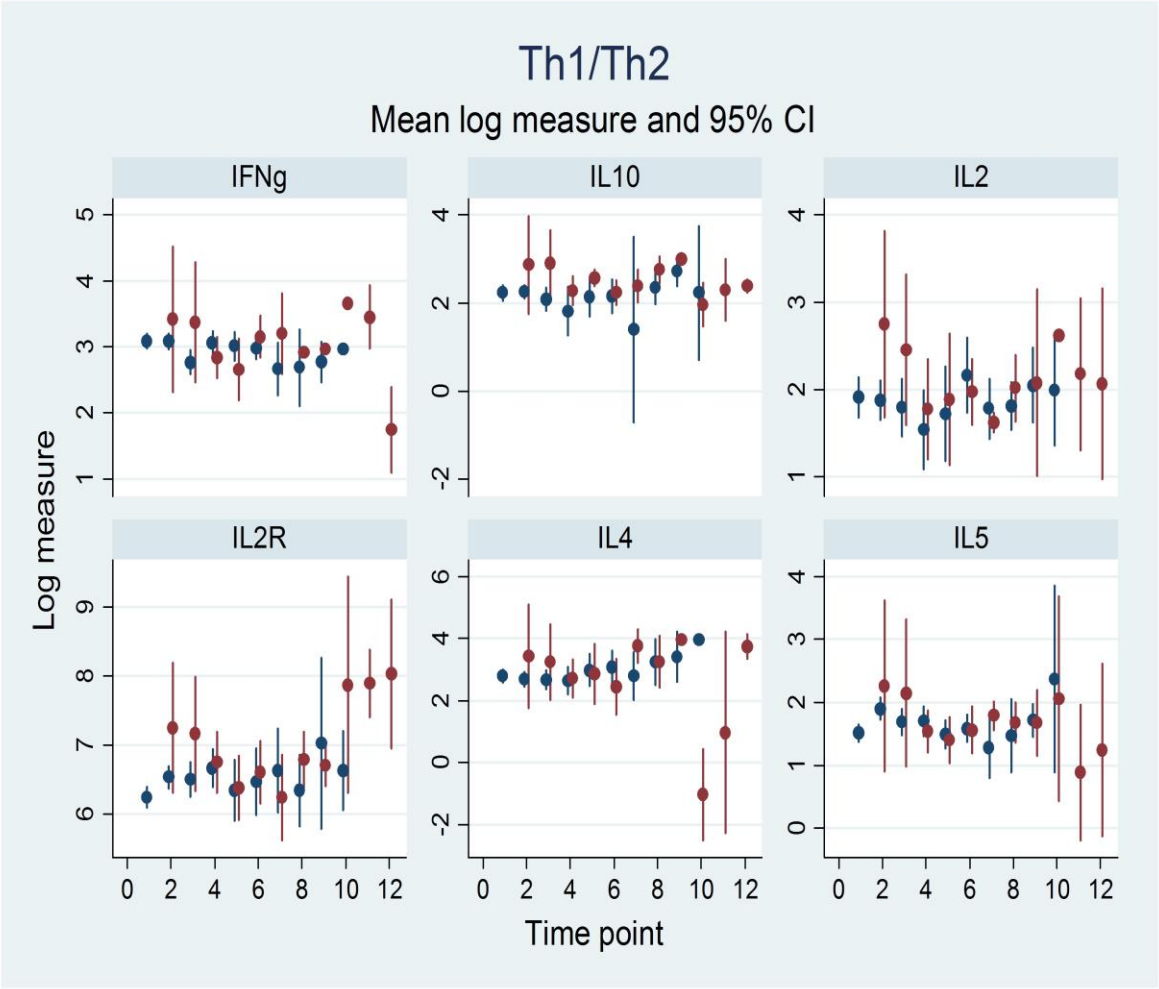


Figure 66: Th1/Th2 cytokine panel at follow-up for IFD (maroon) v no IFD (dark blue)

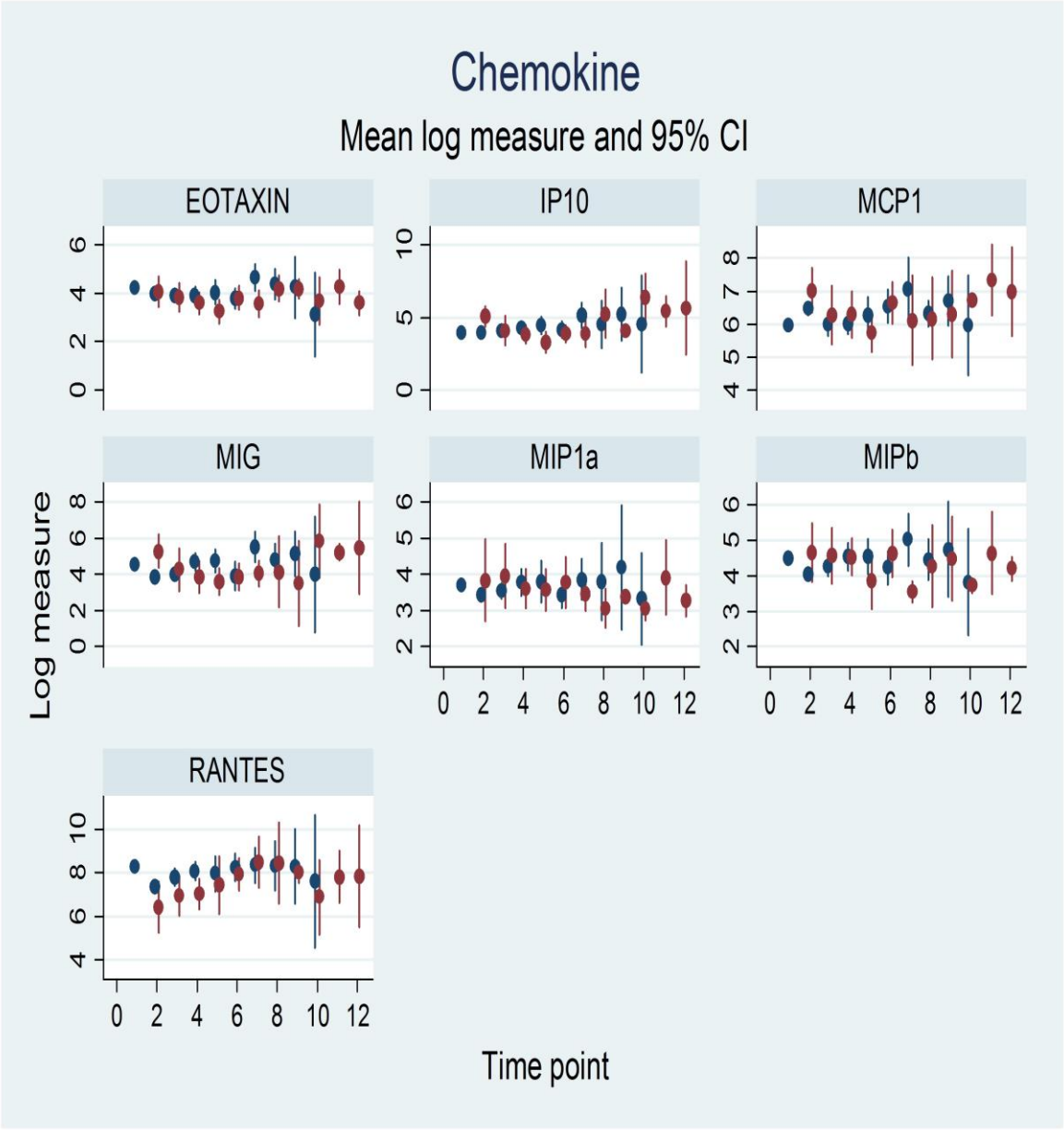


Figure 67: Chemokine panel at follow-up for IFD (maroon) v no IFD (dark blue)

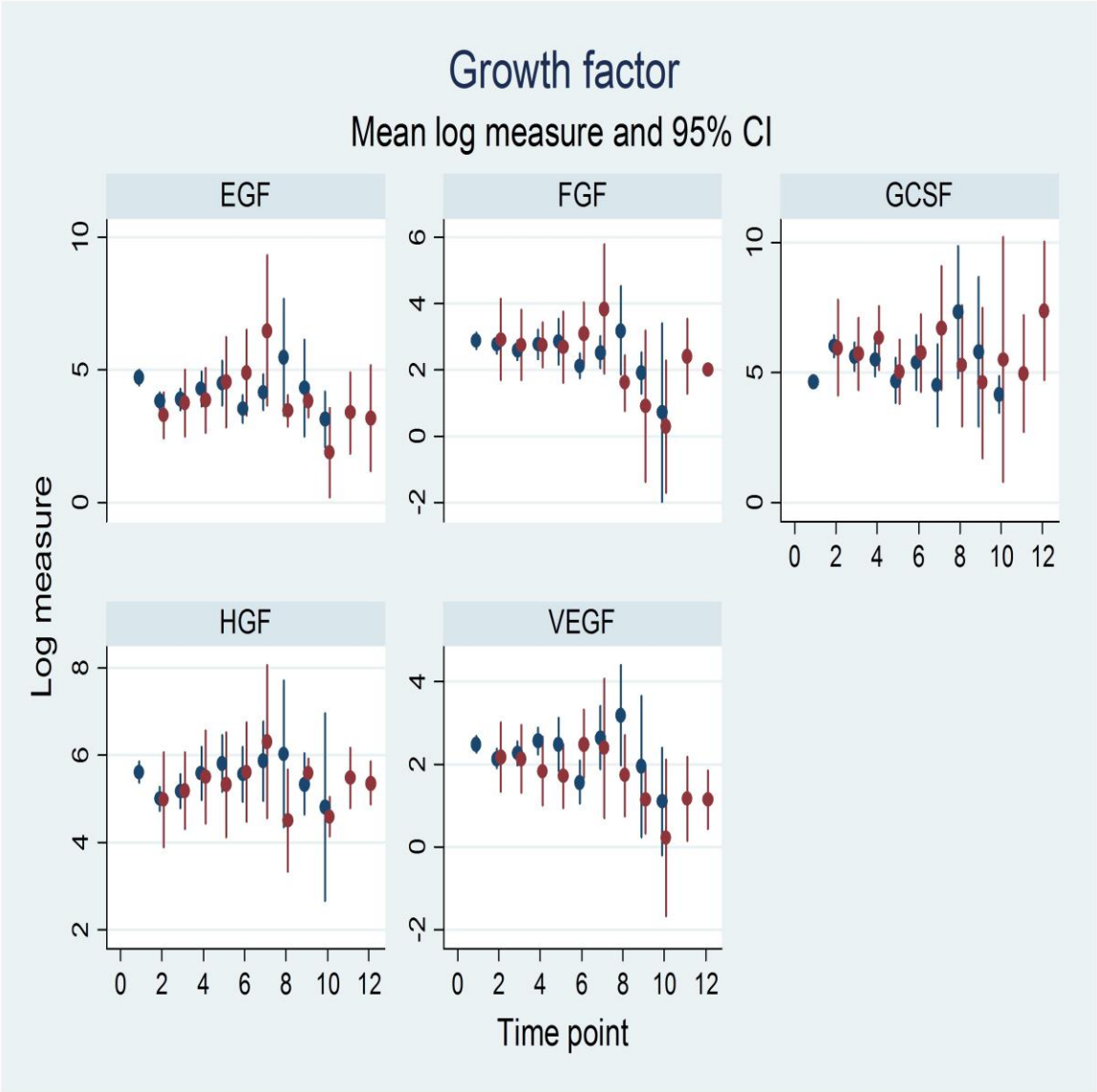


Figure 68: Growth factor cytokine panel at follow-up for IFD (maroon) v no IFD (dark blue)

5.3 Genetic Polymorphisms in genes associated with cytokine biology

A total of 127 patients (median age 53 [range 19-73] years, 76 [60%] male, 83 allografts and 44 autografts, 111 [87%] white Europeans) were tested for 9 SNPs (SNP ID with amino acid change) in TLR1 (rs5743611, 239C→G, T80R), TLR2 (rs5743708, A→G, Q753R), TLR3 (rs3775291, 234C→T, L412F), TLR4.1 (rs4986791, 1363C→T, T399I), TLR4.2 (rs4986790, 1063A→G, D299G), TLR6 (rs5743810, 745C→T, P249S), CLEC7A [dectin-1] (rs16910526, A→C, Tyr238X), CARD9 (rs4077515, 49499C→T, N12S) , and INF-γ (rs2069705, G→A, U/N). In addition, 62 (67%) donors of allogeneic HSCT were also tested for these SNPs. Among the 127 patients 25 were proven/probable IFD, 20 proven/probable IA, 15 possible IFD, 48 not classified, and 39 had no evidence of IFD.

The results are shown in Tables 46-49. No statistically significant differences in any of the genetic polymorphisms were found between IFD or IA cases compared to those with no evidence of IFD or IA (Tables 46 and 47). Among donors of allogeneic HSCT, no SNP was found in TLR 4.2 (rs4986790, A→G D299G) among patients with IFD or IA compared to 4 (36.4%) cases of the those with no IFD or IA (Tables 48 and 49) and this difference was statistically significant.

Table 46 Association between gene polymorphisms and IFD in patients

	IFD	No IFD	P value
TLR1 N=63			
Major allele (C/C) n=57	21 (84.0)	36 (94.7)	0.182
Minor allele (G/G) n=2	2 (8.0)	0	
Major/Minor (C/G n=4	2 (8.0)	2 (5.3)	
Any SNP (C/G or C/G) n=6	4 (16.0)	2 (5.3)	0.156
TLR2 n=58			
Major allele (G/G) n=50	18 (81.8)	32 (88.9)	0.449
Minor allele (A/A)n=0	0	0	
Major/Minor G/A n=8	4 (18.2)	4 (11.1)	
Any SNP (G/A, A/A) n=8	4 (18.2)	4 (11.1)	0.449
TLR3 n=63			
Major allele (C/C) n=40	17 (68.0)	23 (60.5)	0.349
Minor allele (T/T) n=3	0	3 (7.9)	
Major/Minor (C/T) n=20	8 (32.0)	12 (31.6)	
Any SNP (C/T, T/T) n=23	8 (32.0)	15 (39.5)	0.547
TLR4.1 n=62			
Major allele (C/C) n=54	22 (88.0)	32 (86.5)	0.862
Minor allele (T/T) n=0	0	0	
Major/Minor (C/T) n=8	3 (12.0)	5 (13.5)	
Any SNP (C/T or T/T) n=8	3 (12.0)	5 (13.5)	0.862
TLR4.2 n=60			
Major allele (A/A) n=52	18 (81.8)	34 (89.5)	0.401
Minor allele (G/G) n=0	0	0	
Major/Minor (A/G) n=8	4 (18.2)	4 (10.5)	
Any SNP (G/G or A/G) n=8	4 (18.2)	4 (10.5)	0.401
TLR6 n=61			
Major allele (G/G) n=29	13 (54.2)	16 (43.2)	0.484
Minor allele (A/A) n=6	3 (12.5)	3 (8.1)	
Major/Minor (G/A) n=26	8 (33.3)	18 (48.6)	
Any SNP (G/A or A/A) n=32	11 (45.8)	21 (56.8)	0.404

CLEC7A (dectin-1) n=61			
Major allele (A/A) n=51	20 (83.3)	31 (83.8)	0.963
Minor allele (C/C) n=0	0	0	
Major/Minor (A/C) n=10	4 (16.7)	6 (12.2)	
Any SNP (A/C or C/C) n=10	4 (16.7)	6 (12.2)	0.963
CARD9 n=60			
Major allele (C/C) n=15	7 (31.8)	8 (21.1)	0.183
Minor allele (T/T) n=13	2 (9.1)	11 (28.9)	
Major/Minor (G/T) n=32	13 (59.1)	19 (50.0)	
Any SNP (G/T or T/T) n=45	15 (68.2)	30 (78.9)	0.353
INFg n=64			
Major allele (G/G) n=5	3 (12.0)	2 (5.1)	0.566
Minor allele (A/A) n=26	9 (36.0)	17 (43.6)	
Major/Minor (G/A) n=33	13 (52.0)	20 (51.3)	
Any SNP (G/A or G/A) n=59	22 (88.0)	37 (94.9)	0.318

Table 47 Association between gene polymorphisms and IA in patients

	IA	No IA	P value
TLR1 N=54			
Major allele (C/C) n=46	14 (77.8)	32 (88.9)	0.279
Minor allele (G/G) n=0	0	0	
Major/Minor (C/G n=8	4 (22.2)	4 (11.1)	
Any SNP (C/G or C/G) n=8	4 (22.2)	4 (11.1)	0.279
TLR2 n=54			
Major allele (G/G) n=46	14 (77.8)	32 (88.9)	0.279
Minor allele (A/A)n=0	0	0	
Major/Minor G/A n=8	4 (22.2)	4 (11.1)	
Any SNP (G/A, A/A) n=8	4 (22.2)	4 (11.1)	0.279
TLR3 n=58			
Major allele (C/C) n=37	14 (70.0)	23 (60.5)	0.412
Minor allele (T/T) n=3	0	3 (7.9)	
Major/Minor (C/T) n=18	6 (30.0)	12 (31.6)	
Any SNP (C/T, T/T) n=21	6 (30.0)	15 (39.5)	0.476
TLR4.1 n=57			
Major allele (C/C) n=49	17 (85.0)	32 (86.5)	0.877
Minor allele (T/T) n=0	0	0	
Major/Minor (C/T) n=8	3 (15.0)	5 (13.5)	
Any SNP (C/T or T/T) n=8	3 (15.0)	5 (13.5)	0.877
TLR4.2 n=57			
Major allele (A/A) n=49	15 (78.9)	34 (89.5)	0.281
Minor allele (G/G) n=0	0	0	
Major/Minor (A/G) n=8	4 (21.1)	4 (10.5)	
Any SNP (G/G or A/G) n=8	4 (21.1)	4(10.5)	0.281
TLR6 n=56			
Major allele (G/G) n=27	11 (57.9)	16 (43.2)	0.579
Minor allele (A/A) n=4	1 (5.3)	3 (8.1)	
Major/Minor (G/A) n=25	7 (36.8)	18 (48.6)	
Any SNP (G/A or A/A) n=29	8 (42.1)	21 (56.8)	0.299

CLEC7A (dectin-1) n=56			
Major allele (A/A) n=46	15 (78.9)	31 (83.8)	0.655
Minor allele (C/C) n=0	0	0	
Major/Minor (A/C) n=10	4 (21.1)	6 (16.2)	
Any SNP (A/C or C/C) n=10	4 (21.1)	6 (16.2)	0.655
CARD9 n=56			
Major allele (C/C) n=14	6 (33.3)	8 (21.1)	0.289
Minor allele (T/T) n=13	2 (11.1)	11 (28.9)	
Major/Minor (G/T) n=29	10 (55.6)	19 (50.0)	
Any SNP (G/T or T/T) n=42	12 (66.7)	30 (78.9)	0.322
INFg n=59			
Major allele (G/G) n=4	2 (10.0)	2 (5.1)	0.529
Minor allele (A/A) n=23	6 (30.0)	17 (43.6)	
Major/Minor (G/A) n=32	12 (60.0)	20 (51.3)	
Any SNP (G/A or G/A) n=55	18 (90.0)	37 (94.9)	0.481

Table 48 Association between gene polymorphisms and IFD in donors of allogeneic HSCT

	IFD	No IFD	P value
TLR1 N=29			
Major allele (C/C) n=25	15 (93.8)	10 (76.9)	0.191
Minor allele (G/G) n=0	0	0	
Major/Minor (C/G n=4	1 (6.2)	3 (23.1)	
Any SNP (C/G or C/G) n= 4	1 (6.2)	5 (23.1)	0.191
TLR2 n=28			
Major allele (G/G) n=27	15 (100)	12 (92.3)	0.274
Minor allele (A/A)n=0	0	0	
Major/Minor G/A n=1	0	1 (7.7)	
Any SNP (G/A, A/A)n=1	0	1 (5.6)	0.274
TLR3 n=26			
Major allele (C/C) n=14	6 (42.9)	8 (66.7)	0.375
Minor allele (T/T) n=1	1 (7.1)	0	
Major/Minor (C/T) n=11	7 (50)	4 (33.3)	
Any SNP (C/T, T/T) n=12	8 (57.1)	4 (33.3)	0.225
TLR4.1 n=28			
Major allele (C/C) n=23	14 (93.3)	9 (69.2)	0.228
Minor allele (T/T) n=1	0	1 (7.7)	
Major/Minor (C/T) n=4	1 (6.7)	3 (23.1)	
Any SNP (C/T or T/T) n=5	1 (6.7)	4 (30.8)	0.097
TLR4.2 n=24			
Major allele (A/A) n=20	13 (100)	7 (63.6)	0.059
Minor allele (G/G) n=1	0	1 (9.1)	
Major/Minor (A/G) n=3	0	3 (27.3)	
Any SNP (G/G or A/G) n=4	0	4 (36.4)	**0.017**
TLR6 n=26			
Major allele (G/G) n=13	9 (60)	4 (36.4)	0.188
Minor allele (A/A) n=6	4 (26.7)	2 (18.2)	
Major/Minor (G/A) n=7	2 (13.3)	5 (45.5)	

Any SNP (G/A or A/A) n=13	6 (40)	7 (63.6)	0.234
CLEC7A (dectin-1) n=31			
Major allele (A/A) n=27	13 (92.9)	14 (82.4)	0.414
Minor allele (C/C) n=2	0	2 (11.8)	
Major/Minor (A/C) n=2	1 (7.1)	1 (5.9)	
Any SNP (A/C or C/C) n=4	1 (7.1)	3 (17.6)	0.385
CARD9 n=26			
Major allele (C/C) n=12	6 (40)	6 (54.5)	0.760
Minor allele (T/T) n=3	2 (13.3)	1 (9.1)	
Major/Minor (G/T) n=11	7 (46.7)	4 (36.4)	
Any SNP (G/T or T/T) n=14	9 (60)	5 (45.5)	0.462
INFg n=25			
Major allele (G/G) n=6	2 (16.7)	4 (30.8)	0.618
Minor allele (A/A) n=3	2 (16.7)	1 (7.7)	
Major/Minor (G/A) n=16	8 (66.7)	8 (61.5)	
Any SNP (G/A or G/A) n=19	10 (83.3)	9 (69.2)	0.409

Table 49 Association between cytokine gene polymorphisms and IA in donors of allogeneic HSCT

	IA	No IA	P value
TLR1 N=26			
Major allele (C/C) n=22	12 (92.3)	10 (76.9)	0.277
Minor allele (G/G) n=0	0	0	
Major/Minor (C/G n=4	1 (7.7)	3 (23.1)	
Any SNP (C/G or C/G) n=4	1 (7.7)	3 (23.1)	0.277
TLR2 n=26			
Major allele (G/G) n=25	13 (100)	12 (92.3)	0.308
Minor allele (A/A)n=0	0	0	
Major/Minor G/A n=1	0	1 (7.7)	
Any SNP (G/A, A/A) n=1	0	1 (5.6)	0.308
TLR3 n=23			
Major allele (C/C) n=13	5 (45.5)	8 (66.7)	0.414
Minor allele (T/T) n=1	1 (9.1)	0	
Major/Minor (C/T) n=9	5 (45.5)	4 (33.3)	
Any SNP (C/T, T/T) n=10	6 (54.5)	4 (33.3)	0.305
TLR4.1 n=26			
Major allele (C/C) n=21	12 (92.3)	9 (69.2)	0.297
Minor allele (T/T) n=1	0	1 (7.7)	
Major/Minor (C/T) n=4	1 (7.7)	3 (23.1)	
Any SNP (C/T or T/T) n=5	1 (7.7)	4 (30.8)	0.135
TLR4.2 n=22			
Major allele (A/A) n=18	11 (100)	7 (63.6)	0.087
Minor allele (G/G) n=1	0	1 (9.1)	
Major/Minor (A/G) n=3	0	3 (27.3)	
Any SNP (G/G or A/G) n=4	0	4 (36.4)	**0.027**
TLR6 n=24			
Major allele (G/G) n=12	8 (61.5)	4 (36.4)	0.263
Minor allele (A/A) n=5	3 (23.1)	2 (18.2)	
Major/Minor (G/A) n=7	2 (15.4)	5 (45.5)	

Any SNP (G/A or A/A) n=12	5 (38.5)	7 (63.6)	0.219
CLEC7A (dectin-1) n=23			
Major allele (A/A) n=19	11 (91.7)	8 (72.7)	0.296
Minor allele (C/C) n=2	0	2 (18.2)	
Major/Minor (A/C) n=2	1 (8.3)	1 (9.1)	
Any SNP (A/C or C/C) n=4	1 (8.3)	3 (27.3)	0.231
CARD9 n=24			
Major allele (C/C) n=11	5 (38.5)	6 (54.5)	0.718
Minor allele (T/T) n=3	2 (15.4)	1 (9.1)	
Major/Minor (G/T) n=10	6 (46.2)	4 (36.4)	
Any SNP (G/T or T/T) n=13	8 (61.5)	5 (45.5)	0.431
INFg n=23			
Major allele (G/G) n=5	1 (10)	4 (30.8)	0.399
Minor allele (A/A) n=3	2 (20)	1 (7.7)	
Major/Minor (G/A) n=15	7 (70)	8 (61.5)	
Any SNP (G/A or G/A) n=18	9 (90)	9 (69.2)	0.231

Chapter 6 Discussion

6 Discussion

6.1 IFD incidence

The true burden of IFD in haemato-oncology patients undergoing HSCT, intensive chemotherapy or IST is difficult to quantify partly due to imprecise diagnostic tools. In this prospective cohort study a high incidence of IFD was found among patients despite universal prophylaxis, a practice that has been previously shown to reduce the sensitivity of GM.[297] Our results appear not to show this reduced sensitivity. Moreover, the results show the importance of a multi-diagnostic approach in order to improve the diagnostic accuracy of IFD. CT scanning remains the centrepiece for the clinical diagnosis of IPA and the revised EORTC/MSG criteria[10] rely on it as the sole objective clinical criterion for the diagnosis of IPA. Baseline CT abnormalities, both EORTC and non-EORTC signs, were found in 38% of study patients and this was associated with increased risk of IFD. Other important risk factors for IFD found in this study were baseline Karnofsky score <90, baseline cytokines MCP-1 >841 pg/ml and IL-2R >834 pg/ml, monocytopenia >10 days, and bacteraemia. Despite prompt antifungal therapy, proven/probable IFD was associated with higher mortality. IFD was the second commonest cause of death accounting for 20%, with IFD-attributable mortality of 59%.

The EORTC/MSG criteria were designed for use in clinical trials but in this thesis I have demonstrated their applicability and usefulness in daily clinical practice. These criteria represent an important international consensus largely based on expert opinion to define a set of criteria on which clinical trials will be based. Although not designed or validated for routine clinical use, they are arguably the best diagnostic tools currently available in a very difficult area.

The overall incidence rate of 21% and 18% for IFD and IA respectively in our cohort was higher than reported by most previous studies.[88,89,210,211,333] However, our results are closer to post-mortem data by Chamilos et al who performed 1017 autopsies over a 15-year period and found IFD incidence of 31%.[77] The high incidence rate in this study may be due to several reasons.

Firstly, this study was designed to examine the incidence of IFD and IA using the revised EORTC/MSG criteria in routine clinical setting to provide a 'real world' IFD incidence taking due cognizance of the sequential manner in which haematological malignancies and marrow failure syndromes are treated and thus provides an important insight into the patient journey from diagnosis or relapse, through cycles of chemotherapy, which may be followed by allogeneic HSCT and post-transplant complications. Clinical trials such as Marks et al [211] or Wingard et al [210] typically concentrate on single treatment episodes such as allogeneic HSCT. Registry studies such as the Transplant-Associated Infection Surveillance

Network (TRANSNET) [88] are dependent on the completeness of the data collection from the participating centres. Although the overall cumulative incidence of IFD in the TRANSNET study was low at 3.4% there was considerable variation between different institutions ranging from 0-21%. The single centre nature of this study enabled close follow up of patients using all available tools. No patient was lost to follow-up. We were able to recruit 85% of all eligible patients and by so doing able to provide an accurate risk assessment for this cohort.

Secondly, long term follow-up is necessary to truly capture the at-risk period. The typical median follow-up period in clinical trials varies between three[47,207] and six months [210,211]. While the majority of IFD and IA (76% and 78% respectively) in this cohort occurred within 3 months of their index haematological treatment (Figure 35) important differences exist between the subtypes of IFD (Figure 34). Invasive candidaemia occurred significantly earlier (median 17 days) than proven mould disease (median 142 days). Notably all the *Candida spp*. were non-*Candida albicans*, detected in allogeneic HSCT only, reflecting the changing epidemiology in the face of universal extended-spectrum primary prophylaxis with potential treatment implications. The median time to mould IFD of 142 days underscores the importance of prolonged follow-up.

Thirdly the available diagnostic tools are crucial to the accuracy of IFD diagnosis. In this study, using GM or BDG alone would have underestimated the overall incidence of IFD by 10% and 5%, respectively. Most clinical trials or registry studies only rely on GM as the diagnostic tool for probable IFD.[210,211,334] These serodiagnostic tests such as GM, especially with the now widely used GMI cut-off of 0.5, and BDG have improved not only the sensitivity of IFD diagnosis but also provide earlier diagnosis.[290,310] The approved indication for GM is in detecting *Aspergillus* antigenaemia while BDG also detects other fungal pathogens. However, BDG has been shown to be more sensitive than GM for diagnosing IA in line with our results.[312] Unfortunately, it is also associated with high false positive rates.[335] Moreover, there is less experience with BDG and in the UK few laboratories outside the reference laboratories offer this test routinely, not least due to cost considerations. In this study only 38% of probable IFD were dually positive for GM and BDG (Figure 42).

Finally, the gold standard, tissue diagnosis is rarely available in clinical trials and most studies. Biopsy has an important role in the diagnosis of IFD. For example, in this study one of the proven mould cases (morphological appearance of *Aspergillus spp*. by histology but culture negative) would have been misclassified as possible IFD by GM alone in the absence of the subsequent biopsy but was BDG positive. The 16 days interval between the CT scan and biopsy while on treatment dose of liposomal amphotericin B may explain the negative culture results in this case. Species identification was only possible in 2 of the 7 moulds

(Table 19) which may be due to the delay in getting biopsy while patients are on antifungal therapy. However, molecular identification using PCR on biopsy specimens, although not currently an EORTC/MSG criteria, may facilitate species identification and likely to gain wider acceptance in the future.[148,336] Arguably biopsy is more important in providing alternative diagnosis where CT scan and mycological tests suggest IFD. Two cases of probable and two possible IFD cases in this study were shown to have alternative diagnosis.

Postmortem examination provides the final opportunity to secure tissue diagnosis. Certainly the gap between the incident rates of <10% reported in the clinical trials above and the post-mortem rates of 31% by Chamilos et al[77] raises important questions about the accuracy of ante-mortem diagnosis. Here in this thesis I have shown that this gap can be significantly bridged if all the diagnostic tools are utilized. Post mortem (PM) examination was performed in only 5 (2.5%) patients reflecting the declining worldwide trend.[337] Unfortunately two of these autopsies were performed according to UK corner's PM rules (www.justice.gov.uk/whatwedo/coroners.htm), and no biopsies were taken.

Autologous HSCT patients had a significantly lower incidence of IFD than allogeneic or chemotherapy patients similar to previous studies.[72,76,88] However, no significant differences were found between allogeneic, chemotherapy and IST patients suggesting that these treatment modalities have similar levels of

immunosuppression and therefore risk of IFD. However, the risk of IFD varied with cycles of chemotherapy. Induction chemotherapy accounted for 76% of all chemotherapy-related IFD while consolidation and aplasia post consolidation accounted for 19% and 5%, respectively in keeping with previous observations.[73]

Seven patients in this cohort had more than one IFD episode. Two of the patients had 2 episodes of probable IFD 6 months apart while the other had probable IFD and then proven mould NOS 13 months apart. In both of these cases the patients achieved radiological CR according to MSG/EORTC response criteria [321] after the first IFD. Another patient had 2 possible IFD episodes 11 months apart after achieving CR. The four remaining patients had possible, probable, and proven IFD followed by candidaemia and *P. jirovecii* in separate febrile neutropenic episodes.

Bacteraemia was seen in 36% of patients and was concomitant with proven/probable IFD diagnosis in 9% of cases. No difference in concomitant bacterial infections were found between allograft, autograft and chemotherapy patients (Table 15) in contrast to the findings of Bergeron et al who found this in 48% of allogeneic HSCT recipients but only 5% in non-transplanted acute leukaemia patients.[333] That study was a smaller study (N=55) and used a combination of original and revised EORTC/MSG criteria which makes comparisons with our study difficult. In addition, bronchoscopy was only

performed in only 13 cases in our study and the concomitant infections refer to bacteraemia rather than presence of bacteria from BAL. Arguably bacteraemia is a more significant pathology as blood is normally sterile in contrast to the respiratory tract.

The low rate of bronchoscopy for diagnostic purposes was due to unavailability of this service in a timely fashion. It was done only thrice a week with a waiting list making it difficult to fit patients in at a short notice. Positive bacteriological cultures from BAL were recovered from 3 patients (Table 25). Baseline pre-therapy bronchoscopy was not accepted by most patients. In fact, the initial inclusion of this as part of the study for all transplant patients led to a significant reduction in recruitment. Consequently a change of protocol was adopted for BAL to be optional.

6.2 Diagnostic tests

6.2.1 GM & BDG

GM is an established diagnostic test for IFD. However, the dynamics of GM release during infection remains unclear.[287,294] It is therefore necessary to conduct regular surveillance monitoring during at-risk period to maximize the chance of early detection. In this study 3086 tests were carried out. Surprisingly only 5% of these tests were positive and only 2% of these were true positives that

were temporally related to clinical signs of proven/probable IFD while the rest were unclassified. The false positive rate among proven/probable IFD cases was 18%. Using proven IA and BDG-only positive probable IFD as true IFD, the sensitivity, specificity, PPV, and NPV were 54%, 71%, 82%, and 39%, respectively. The wide variation in the performance of GM even in studies using GMI cut-off of 0.5 is well recognized.[338] The lack of tissue diagnosis, the true gold standard, makes it difficult to define the disease state. The low sensitivity in this study may reflect the inclusion of probable IFD with proven disease. Proven disease in this study was small.

The revised EORTC/MSG criteria recognizes positive GM results that bear temporal relationship with clinical and host factors but does not specify the minimum number of positive results in order to fulfill the diagnostic criteria for probable IFD.[10] Eight of the 22 cases (36%) who had positive GM in this study had positive results more than once (Figure 41). Of these 8 cases 6 died due to IPA (5) or relapsed AML with active IPA (1). The two cases that survived became GM negative and achieved a complete radiological response suggesting that persistent GM positivity is associated with poor prognosis in as previously reported.[92,296]

6.2.2 PCR & LFD

Lack of standardization remains the main barrier to molecular diagnostic tests such as PCR. The consensus PCR is an attempt to achieve international

standardization.[317,339] It was performed on 323 EDTA samples from 52 patients with proven/probable IFD and a selection of possible and no evidence IFD cases. Using a Ct cut off of <50, 19 (3%) samples were positive. However, the Ct of these positive samples ranged from 36-49 suggesting weak-moderate positivity. Using the consensus PCR as mycological evidence it would have truly identified 3 (6%) probable IFD. All these 3 cases were also GM positive earlier or at the same time as PCR and therefore PCR offers no additional advantage over GM. Serum PCR appears to have better sensitivity and PPV, the key diagnostic variables in this cohort, than the EDTA whole blood method (Table 28).

The immunochromatographic LFD is a new serodiagnostic tool for IA not yet validated for use in clinical practice. 973 samples from patients with full EORTC classification spectrum from proven to no evidence of IA were tested (Table 24). The sensitivity of this test is poor (9%) for detecting proven mould and GM-positive probable IA (Table 25). This may be partly due to the narrow antigen-specificity, derived from the hyphael tip of growing *Aspergillus spp.*[313], of this test. However, it was negative in all 5 sera from proven *A. fumigatus* patients. It is also worth noting that the LFD is a qualitative test. Of the 35 positive samples 10 were strongly positive (from 2 patients with proven mould NOS, and probable IA), 11 were moderately positive (from 3 patients: 1 mould NOS and 2 probable IFD), and 14 were weak positive (6 patients with proven mould, probable, and possible IFD).

The narrow specificity of the LFD may be a disadvantage in clinical practice where the primary focus is in identifying pathogenic moulds. The actual species may only be relevant where differing antifungal sensitivities of the different organisms may be an issue. This requires identification by mycological cultures and susceptibility testing.[336] Only 9% of cases were identified by both GM and LFD (Figure 43). This was rather surprising given LFD specificity for *Aspergillus spp*. On the contrary there was a better relationship between BDG, a panfungal assay, and LFD (Figure 44). In fact, all the patients identified by LFD as true probable IFD were also detected by BDG suggesting that LFD has no additional diagnostic advantage over BDG.

6.2.3 Radiology

CT scanning remains the centrepiece for the clinical diagnosis of IPA and the revised EORTC/MSG criteria rely on it as the sole objective clinical criterion for the diagnosis of IPA.[10] Baseline CT abnormalities of EORTC and non-EORTC signs were found in 38% of patients. These abnormalities were associated with increased risk of IPA in patients with EORTC signs. Baseline CT scanning was performed pre-therapy with no signs of sepsis, vividly illustrated by two case histories in Figures 51 and 55. Patients with baseline abnormalities were similar in demographic and clinical characteristics to those with normal scans. CRP and neutrophil counts did not differ not only between these 2 groups but also between those with EORTC and non-EORTC signs. No significant differences were found between patients who developed proven/probable IFD compared to those who did not in patient with EORTC signs in the distribution, morphology and location of

signs. The demonstration of baseline abnormalities in non- neutropenic patients with no signs of sepsis is intriguing and to our knowledge not previously described in a prospective study among adult haematology patients. A retrospective study in children found abnormal chest CT scan in 44% of children prior to undergoing HSCT.[340]

Fever-driven management strategies, such as ours, depend on failure to respond to broad-spectrum antibacterial therapy for ≥96h to diagnose IA. Fever is not currently recognised either as a host or clinical factor in the current revised definitions.[10] However, it remains the centrepiece for empiric therapy, widely endorsed by international guidelines for treatment of IA.[4,11] Patients who are otherwise well with no fever and abnormal CT findings present clinical challenge for the treating physician. Positive GM or BDG before the onset of fever is well recognized[242,341] but abnormal CT preceding other clinical signs of infection and mycological evidence of IA is not.

In a retrospective study Nucci *et al* described a group of patients with non-EORTC signs who on subsequent scanning exhibited the typical EORTC signs after a more prolonged neutropenic phase.[342] They suggested that the non-EORTC signs may be an earlier clinical manifestation of IPA before the onset of neutropenia. In this thesis I found no significant differences in baseline neutrophil, lymphocyte, or monocyte counts suggesting that this did not influence the

prevalence or the nature of the lesions in this study (Table 36). Moreover, time to first febrile episode and IA was not statistically significantly different.

IFD is a disease of the immunocompromised, often due to underlying haematological malignancy, and its often complex treatment regimens and many risk factors have been described.[49] Abnormal baseline CT scan adds to this list reflecting the significance of damaged lungs in the pathogenesis of IPA. Indeed the respiratory tract is in constant contact with *Aspergillus* conidia, saprophytic organisms which are able to exploit the immunocompromised environment proffered by the failure of the immune system.[343] Neutrophils and macrophages in concert with intact respiratory mucosa provide the primary effector cells against invasive disease.[51,95,97] It is likely that pre-existing lung abnormalities may provide the type of ecological milieu conducive to fungal proliferation.

Nodules are by far the commonest type of lesions seen in the diagnostic scans in this study in keeping with previous observations in acute angioinvasive IPA.[149,344] The halo sign was seen in about 44% of IPA patients. Nearly 50% of lesions were bilateral reflecting the multifocal nature of IPA. All but one of the patients with baseline EORTC-signs at time of diagnosis of IPA had the diagnostic lesions in the same lobe, while three quarters occurred in a different lobe of the same lung suggesting angiogenic spread from the original focus. In contrast diagnostic lesions in patients with non-EORTC signs were predominantly (78%) contralateral

but significant proportion (44%) occurred within one lung. Eight of these 9 lesions were nodules reflecting the general trend. The relatively fewer cavities/air crescent signs in this prospective study are in keeping with early scanning policy and consistent with previous studies.[149,344]

Despite the good clinical performance of CT scan lesions, especially EORTC-signs performed early in the process to correctly identify IPA cases, CT remains observer-dependent and non-specific.[47,345] It is therefore imperative that imaging is used in conjunction with other tests such as GM, BDG and biopsy whenever possible. The agreement between the 2 radiologists was good (Kappa >0.69) for EORTC signs but less so for non-EORTC ones. It is worth noting that the observers in this study were experienced radiologists with expertise in IPA. Even so 4 (8%) of the diagnostic scans with EORTC signs would have been misclassified using a single chest radiologist.

The hallmark of angioinvasive aspergillosis is coagulative necrosis with surrounding area of haemaorrhage due to thrombosis of the affected vessels and effective sequestration of the infected area.[53,143,145] The hypothesis for contrast studies was that this process is relatively avascular and therefore should have no or reduced enhancement in contrast to malignant lung nodules where angiogenesis is a key pathogenic mechanism.[277] Twenty-seven patients were studied and the peak enhancement post contrast injection for both the

background lung and the lesions of interest was at 2-3 min. No significant differences were found between patients with proven/probable IPA and those with consolidation (Table 40). This was disappointing but may be due to two main reasons.

The first reason is that the number of cases was small which makes meaningful comparison difficult. Secondly, the comparator arm to proven/probable IFD was consolidation which may not be appropriate as IPA may present with consolidation.[149,346,347] Moreover, 4 of the 6 patients with consolidation were also GM-positive, which is considered by some authors as probable IFD.[333,342] This is a fundamental point as, unlike lung tumours which may be benign or malignant, there is no clear cut comparator arm in lung infection except biopsy proven alternative sources of sepsis with no evidence of IPA. Nonetheless, IPA nodules enhance with contrast. This would suggest that there is no decrease in blood flow to the area of interest even with thrombosis of some of the vessels. Perhaps a compensatory hyperaemia which occurs as normal inflammatory response is responsible for this enhancement.

Systematic CT scanning is not without its own drawbacks. The average number of scans/patient in this study was 2 but a typical febrile patient may have 4 or more. This undoubtedly involves more radiation and there are growing concerns about the risk of future secondary malignancies.[348] Equally the importance of CT

scanning in the diagnosis of IPA is not in doubt. In this study steps were taken to reduce radiation exposure by using low-dose scanning techniques which would not compromise diagnostic accuracy.[349]

Practical difficulties remain in assessing response to antifungal therapy in routine clinical practice and studies thereof such as this. Of the 48 diagnostic scans only 17 (35%) and 13 (27%) patients had follow-up 1 and 2 respectively. Early discharge after engraftment and resolution of fever contributed to 29% of failure to objectively assess response to antifungal therapy. Radiological CR was rare in this cohort; achieved only in 1 of 17 follow-up 1 and 2 of 13 follow-up 2 scans. Progression of radiological lesions was seen in 4 patients at follow-up 1: 2 deteriorated further and died of IPA, while the other 2 achieved CR and PR, analogous to the previously reported worsening of radiological lesions around 2 weeks in the face of clinical improvement.[344]

6.2.4 Biopsy

CT scanning is a crucial diagnostic tool for detection of IFD. However, CT lacks specificity even for the highly suggestive nodule with halo sign.[350,351] The specificity is improved by serodiagnostic tests such as GM and BDG but obtaining tissue diagnosis is the gold-standard for the diagnosis of IFD. Previous studies on tissue diagnosis were largely retrospective and small. Kim et al performed a retrospective analysis on 32 cases with haematological malignancies with CT features suggestive of IFD on whom they performed open lung biopsies.[268] They

found 17 cases of histologically proven IFD among their study population (53%). Complication rates in this study were said to be minimal with no mortality. Nosari et al used the percutaneous CT-guided approach in a retrospective study involving 17 cases in 5 years and found Aspergillus spp. in 8 and *mucorales* in 4 cases. [278]

In this study 38 biopsies involving 60% of eligible patients were performed. In the 40% of cases where biopsy was not carried out, this was mainly due to interference with chemotherapy/transplant schedules or lack of theatre time when needed. Physician reluctance to biopsy neutropenic and thrombocytopenic patients also played a part. Biopsy results were useful in 14 cases (37%) where the diagnosis was proven (group A) or an alternative diagnosis was made (Group B). The quality or adequacy of the samples obtained from the biopsy was the key factor that determined the usefulness of the results. In groups A and B all samples were adequate compared to 33% in group C ($P<0.001$). The inadequate biopsies were all lung biopsies obtained by VATS (14), CT-guided (1) and transbronchial (1) approaches. The VATS approach is minimally invasive but accurate localisation of the lesion may be an important limitation which may give false negative or inconclusive results. Methylene blue tattooing did not improve this, although the number of cases where this was performed was small.

Tissue biopsies are not only important in the diagnosis of IFD but also for treatment based on the correct species identification and susceptibility testing.[336] The number of cases with proven diagnosis in this study was surprisingly low given the highly selective nature of susceptible patients with clinical features compatible with IFD who had GM and BDG surveillance performed twice weekly. The sensitivity of biopsy was low at 35% in accordance with previous studies.[352] Only two of the seven proven cases were culture positive. Earlier biopsy may improve culture positivity. Molecular detection techniques such as PCR are not currently utilised in species identification in the revised EORTC/MSG criteria but they are likely to be used in the future.[327,336] Four of the five proven IFD biopsies in this study were positive by PCR and in two of these species identification was possible by nuclear ribosomal repeat sequencing suggesting a complementary role with culture.

It can be argued that biopsy is more important in providing alternative diagnosis where CT scan and mycological tests suggest IFD. Two cases of probable and two possible IFD cases in this study were shown to have alternative diagnosis. The 15 days median interval between probable/possible IFD and proven cases in this study reflects challenges such as physician reluctance to biopsy neutropenic, thrombocytopenic patients, and timely availability of theatre slots. Species identification was only possible in 2 of the 7 moulds which may be due to the delay in getting biopsy while patients are on antifungal therapy.

The adverse events from biopsy were reassuringly low. This is an overriding concern for patients and physicians looking after them. Surgical techniques such as VATS are safe and where available should be used as part of the diagnostic armamentarium. The biggest challenge facing such techniques is whether adequate samples can be obtained.

6.2.5 Antifungals-prophylaxis, treatment and role of TDM

Universal prophylaxis initiated at admission was the local policy and the agent of choice was based on the perceived risk of neutropenic sepsis. There was considerable alteration thereafter based on the number and frequency of neutropenic sepsis and response to antimicrobial therapy. It therefore, becomes very difficult to assign a particular agent to break-through IFD. The agent that a particular patient was receiving within 2 weeks of EORTC classification may be a useful surrogate marker for this. Based on these the break-through proven/probable IFD was 22%, 17%, and 13% for posaconazole, itraconazole, and fluconazole, respectively. As these antifungal prophylactic drugs were used in different risk strata, these rates are not directly comparable. It is also worth noting that about 75% of posaconazole and voriconazole were not given according to protocol (Table 21).

Antifungal therapy was received by 50% of study patients during the course of the study. Although this may seem excessive in a cohort with proven/probable IFD

incidence of 21%, a closer look at patients who had therapy for two weeks or more revealed a figure of 33% which bears close resemblance to the autopsy data.[77] Moreover, the combined incidence of proven, probable, and possible IFD was 34%. Possible IFD with antibacterial-refractory neutropenic sepsis is usually treated in the same way as proven/probable IFD in clinical practice.

As azoles form the core of antifungal prophylaxis in our cohort, empiric therapy is usually with polyene (liposomal amphotericin B) or echinocandins (caspofungin) in accordance with ECIL-3 guidelines on antifungals.[11] Azole prophylaxis followed by azole treatment was rare in this cohort (9%).

The role of therapeutic drug monitoring (TDM) was assessed by a parallel study firstly to develop this capacity locally and secondly to use this service to guide clinical care.[353] For itraconazole, a target serum itraconazole concentration of 0.5 mg/L is recommended for effective prophylaxis.[354,355] A large inter-patient variability in the measured serum itraconazole and hydroxyitraconazole concentrations (0.02-5.31 mg/L and 0.02-4.42 mg/L for itraconazole and hydroxyitraconazole, respectively) was found. Moreover, 70 of the 163 samples (43%) had serum concentrations below 0.5 mg/l.[356]

Perhaps TDM is more important for posaconazole as its pharmacokinetics is affected by fatty food intake and proton pump inhibitors, which are commonly prescribed in haematology patients undergoing chemotherapy or HSCT.[355] Of the 57 samples from patients prescribed posaconazole, 30 (53 %) had pre-dose serum concentrations below the threshold concentration of 0.5 mg/L suggested by Andes *et al*.[355] and 43 (75 %) had concentrations below the FDA-recommended minimum concentration of 0.7 mg/L (http://www.fda.gov/cder/foi/nda/2006/022003s000_NoxafilTOC.htm).[356] This is in keeping with previous observations,[357,358] highlighting the difficulty in achieving serum concentrations considered appropriate for antifungal activity in most patients. This is quite worrying and may partly account for the high incidence of IFD in this cohort. Subsequently attempts have been made to ensure adequate concomitant fatty food intake for patients on posaconazole.

6.3 Cytokines

Predicting IFD prior to chemotherapy or HSCT can allow a more stratified antifungal prophylaxis and treatment. Baseline serum cytokine measurement, made possible by the availability of bead technology, provides useful measure of immune function. Four serum cytokines, MCP-1, MIP-1α, IL-15, and IL-2R were found to be significantly higher, while IL-4 was significantly lower in patients who developed proven/probable IFD compared to those with no evidence of IFD. A smaller IA cohort showed higher HGF, MCP-1 and RANTES in proven/probable IA compared to no IA.

Cytokines and chemokines, produced by an array of immune cells, form an important component of host defenses against infection by various pathogens. Typically their actions are brief and self-limited with inhibitory feedback mechanisms to turn down their responses.[132] The presence of significant differences between those who develop IFD and those who remain disease free during the course of significant, often prolonged, immunosuppressive therapy would suggest that these baseline cytokines signify an important immune dysregulation or that they might be a marker for occult infection. The respiratory tract is in constant contact with *Aspergillus* conidia, which exploit the immunocompromised environment proffered by the failure of the immune system.[343] Neutrophils and macrophages in concert with intact respiratory mucosa provide the primary effector cells against invasive disease.[51,95,97] MCP-1 (CCL2), MIP-1α (CCL3), and RANTES (CCL5) are important chemotactic molecules for neutrophils, monocytes, and dendritic cells.[132,359,360] If these are markers of occult infection then they represent pretty early biomarkers preceding fever, CRP or indeed IL-6 rise.

IL-4 is a Th2 cytokine and high levels are associated with adverse outcomes in murine models of IFD.[361] The lower levels in proven/probable IFD may be a marker of ongoing Th1 inflammatory reaction. IL-15 and IL-2 are important immunoregulartory molecules of the innate immune system and share two of the components of their receptor molecules, IL-2Rβ, IL-2Rγ.[132,361] High levels at baseline is likely to be a marker for occult infection.

It is important to note that 31 patients were excluded from this baseline analysis who either had fever or their chemotherapy given prior to sample collection. In doing so major causes of inflammatory were avoided. Care was taken to transport the samples on ice and ensure sera were separated immediately and frozen at -80^{0}C until ready for analysis.

Follow-up cytokine assays were performed on 412 samples with a median of 2 samples per patient collected on a fortnightly basis. No significant differences were found between patients with proven/probable and no evidence of IFD. Could it be that a more frequent sampling is required to clearly see a pattern of cytokines that differentiate between IFD v no evidence of IFD? It may also reflect the fact that cytokines are mainly an innate immune response and less of an adaptive immune system and as such lack specificity for fungal infection at a time when patients are having a massive inflammatory challenge from chemotherapy, donor cell infusion, as well as fever from non-IFD cases.

Chai et al investigated 119 patients with IA from the Global Comparative Aspergillosis Study[47] and found high baseline IL-8 and persistently elevated IL-6 and CRP as poor prognostic factors.[362] Important differences exist between that study and the current one. Firstly the Global Comparative Aspergillosis Study was a clinical trial comparing voriconazole v amphotericin B and the case

definitions for proven and probable IA were based on earlier EORTC/MSG 2002 definitions[9]. Secondly there was no comparator arm in that trial as the study entry criteria was proven/probable IA. It is therefore difficult to assign the cytokine dynamics to IA. Thirdly the baseline values in that study refer to IA prior to initiation of antifungal therapy which is contrast to true baseline values in the current study where the patients showed no clinical signs of sepsis or chemotherapy. Finally, the persistently high CRP and IL-6 after initiation of antifungal therapy obviously mean that the affected patients are not responding to therapy. It is interesting that there was no mention of fever in the Chai et al study, a well-known clinical indicator of sepsis which correlates with CRP. In this thesis duration of fever was found to correlate with magnitude of CRP (Figure 11). Persistent fever despite antifungals or antibacterial for that matter is an indication to alter therapy especially if this was the case for a week or more as in the Chai et al study[362]. This is especially relevant in a disease which is difficult to diagnose and the majority of patients were probable cases some of which would be unclassified on the revised EORTC/MSG definitions.

6.4 Risk factors

This cohort was selected on basis of traditional risk factors[38,49,174] especially prolonged neutropenia. Severe neutropenia $<0.5x10^9$/L was seen in 93% of patients and 59% had severe neutropenia for 10 days or more. It is therefore, not surprising that no significant association was found between neutropenia and IFD on multivariate analysis. Abnormal baseline CT scan, prolonged monocytopenia,

bacteraemia, poor performance status and high serum MCP-1 and IL-2R are independent risk factors described here.

Abnormal baseline CT reflects the significance of deranged pulmonary milieu in the pathogenesis of IPA. Other baseline independent risk factors include high MCP-1, high IL-2R, and Karnofsky score of less than 90 reflecting the importance of pre-therapy clinical status as risk factors for IFD. High MCP-1 and IL-2R are likely to reflect occult infection. Because no normal ranges exist for the normal population for these cytokines, the interquartile ranges were used instead. High levels refer to levels higher than 419 pg/ml and 841 pg/ml for MCP-1 and IL-2R, respectively.

Karnofsky score was first described by Karnofsky and Buchenal in 1949 as a tool to assess a patient's ability to survive chemotherapy.[363] Since then it is has been widely used to assess risk of death in cancer patients with fungaemia[364], risk factor for bacteraemia[365,366], and prognosis in BMT[367,368]. It is a semi-quantitative measure of patients' clinical state but remains somewhat subjective as are most clinical assessments. A more specific performance score for allograft patients, PAM score, was not found to be a significant risk factor IFD in this study.

Prolonged severe monocytopenia, especially in concert with neutropenia renders the affected patients bereft of the necessary phagocytic function required to combat inhaled spores and thus prevent invasive disease. Monocytopenia has been noted in previous studies as a risk factor for IFD.[35]

It is not infrequent for bacterial infection to coexist with IFD.[81,333] The association is not surprising given the underlying immune paresis. It is vitally important to demonstrate this as it requires dual antibacterial and antifungal therapy. To my knowledge bacteraemia has not been previously demonstrated as an independent risk factor for IFD. The infection does not have to be concomitant and the organisms may be Gram negative or positive. It is likely that bacteraemia anytime during the at-risk period is a surrogate marker for the degree of immunodeficiency. Alternatively there be could a more fundamental biological interaction between bacteraemia and the immune system which facilitates IFD.

Previous studies have found GVHD and steroids as risk factors for IFD.[30,35,38,40,50] In contrast to earlier studies there was only a trend in the multivariate model for GVHD as a risk factor (HR 2.45; 95% CI 0.97-6.24; P=0.059) in this study. This is likely to be due to smaller sample size in this study (N=99 allogeneic HSCT) but more importantly GVHD was seen in 37% of allografts but only 9% had grade III-IV acute GVHD and 7% had extensive GVHD. This is much lower than the case in the above studies and reflects the differences in transplantation practices.

Reduced intensity conditioning with T-depletion, using anti-thymocyte globulin (ATG) or alemtuzumab were used in our allograft protocols. Alemtuzumab is associated with profound and prolonged immune suppression.[30] On the other hand Alemtuzumab or ATG significantly reduces the risk of severe form of GVHD, an important risk factor for IFD.[49] Consequently steroids were only sued in 26% of transplant patients and therefore not surprising that it was not found to be a significant independent risk factor.

Genetic polymorphisms involving genes of the innate immune system are important risk factors for IFD and there is a growing list of these candidate genes.[62,66,69,121,122,324] The selection of 9 genes to examine for this susceptibility was based on these studies. No statistically significant associations were found between these previously described SNPs and susceptibility to IFD in our cohort. The reason for this is likely to be due to sample size and the small number of IFD cases in the cohort.

The number of IFD cases in our cohort was quite small (25 proven/probable cases) which makes the detection of these associations statistically challenging. The cases examined were based on the allogeneic and autologous HSCT patients whose DNA samples were collected for chimerism studies in the allogeneic HSCT programme in our institution. Our study was not initially set up to examine this relationship and DNA samples were not stored for this purpose.

Similarly the donor samples were provided from the chimerism studies and were not available or sufficient in all cases.

6.5 OS and Prognostic factors

In this cohort 64 patients died at a median follow-up of 18.5 months (95% CI 17.3-19.8). Relapse of the primary haematological malignancy was the commonest cause of death followed by IFD which accounted for 20% of all deaths. However, assigning a cause of death may not be straight forward as there may be competing causes such as relapse and IFD. No general consensus guidelines exist but for myeloablative allogeneic HSCT patients an American Society for Blood and Marrow Transplantation (ASBMT) has agreed on a scheme that helps consistency of reporting.[369] Although this is followed here it must be recognized that there is an inherent bias towards relapse and GVHD at the expense of infection; sepsis is only accepted as cause of death in the absence of relapse and active GVHD.

Among the 40 IFD patients in the study 22 (55%) died at a median of 25 days from IFD diagnosis; 13 died due to IFD in remission of hematological malignancy and no GVHD and therefore attributable mortality of 59% consistent with previous studies.[46,90,91] However, 5 of the 6 relapse cases had active IFD and therefore the true attributable mortality rate in this cohort was 82% (18/22). The adverse outcome for IFD cases was not due to late diagnosis or delayed initiation of

therapy as antifungals were commenced with clinical suspicion of IFD while investigations were carried out; a hybrid empiric and pre-emptive or diagnostic-driven approach[242]. Although the outcome for IFD patients has been improving steadily in the last 30 years[8] this study has high-lighted the fact that for patients with proven/probable IFD using the current revised EORTC/MSG criteria the prognosis remains poor. This is likely due to advance disease in this category exemplified by the fact that all the proven mould cases in this cohort died, although one died from PTLD rather than IPA. It is also worth noting that the changes in the diagnostic criteria for IFD over time make it difficult to compare older studies to more recent ones such as this one.

Interestingly patients with possible IFD or not classified had intermediate outcome which may reflect the heterogeneous nature of this group. The challenge is to separate out true IFD cases from those with other non-IFD pathologies or false positive mycological test using better diagnostic tools. In practice this is a large group compared to proven/probable IFD.[370] Possible IFD and those unclassified on the basis of non-EORTC radiological signs with no alternative diagnosis are in practice treated as proven/probable IFD with antifungals and some recent studies consider these as *bone fide* IFD.[333,342] The intermediate outcome of these cases suggests that they may represent an earlier form of IFD. If so what then determines progression to full blown proven/probable disease? In this cohort it was certainly not due to delayed institution of antifungal therapy.

In the multivariate model the independent factors associated with mortality for the whole cohort were ITU admission, having allogeneic HSCT/chemotherapy/IST v autologous HSCT, AML/MDS v other diagnosis, bacteraemia, and hepatic dysfunction. These are collaborated from previous studies.[46,90,93] Excluding the possible and unclassified cases ITU admission, allogeneic HSCT/chemotherapy/IST v autograft, proven/probable IFD, and renal impairment with creatinine >240 were significant independent factors. Persistently positive GM results were only significant in the univariate model in line with Koo et al[92] but not in the multivariate analysis probably due to small numbers.

6.6 Case discussion

Case 2: This 61 year old lady had allogeneic HSCT from unrelated donor with DQB1 mismatch for poor risk AML and had several important post-transplant complications. Her baseline CT showed tree-in-bud appearances in the RLL and was clinically well (afebrile, CRP <5 mg/L, Karnofsky score 90, PAM score 29, Hb 12.1, Neutrophils 6, platelets 391, marrow was in CR). However, within 24 hours of starting conditioning chemotherapy she became febrile and was started on broad-spectrum antibiotics. This was initially thought to be due to 'campath fever' commonly seen following administration of first dose of alemtuxumab and may be related to cytokine storm or acute phase reaction from the drug.[371] Her temperature settled with antibiotics and culture results were negative.

Her diagnostic scan was done 22 days after admission into study and it showed GGO. A subsequent non-study clinical scan a week later showed that the RUL GGO had progressed to consolidation. GM and BDG remained negative. Second diagnostic scan 3 months after the first diagnostic scan was reported as:

'There are new tiny (<2mm) focal opacities in the right upper lobe…..Findings are non-specific but not typical for established angioinvasive aspergillosis'

Subsequent review by study radiologists confirms nodules in RML. GM and BDG were positive during 7 days prior to CT scan. This highlights the importance of chest radiologist review.

Her antifungal management (Figure 13) deserves some comments. She was on antifungals for a total of 272 days of her 296 study days costing £29,711.60. The rationale for changing antifungals was not clear especially from posaconazole to itraconazole. This was an outpatient decision and may be due long duration of posaconazole (120 days!). However, she still had ongoing issues with recurrent CMV reactivations, cytopenias, active extensive GVHD of skin and gut and on immunosuppressive therapy. TDM on 2 occasions (retrospectively) showed that her serum posaconazole and itraconazole levels were sub-therapeutic at 0.2 mg/L and 0.18 mg/L, respectively. TDM should form a crucial component of such patients in the future but at the time of the study this was not available locally.

The difficulty in proving IFD diagnosis is exemplified in this case. The interval between probable and proven IFD was 168 days. She became a proven case at post mortem. The PE, ongoing diarrhea, chest pain, and reduced conscious level on her last admission before death were not connected to IFD ante-mortem. Only post mortem examination could have proven this case and the extent of the disease was colossal. Unfortunately, the post mortem samples were culture negative and tissue PCR failed and she remained a proven disseminated mould disease NOS.

Case 7: This was another case of disseminated aspergillosis with biopsy proven cardiac involvement post allograft. Her baseline CT chest showed GGO but her immediate post- transplant period was relatively straightforward. Prior to her allograft she was treated with alemtuxumab for her p53 deleted CLL. Her allograft was complicated by gut GVHD, CMV and adenovirus reactivations, prolonged cytopenia, and recurrent bacteraemias. In the late post-transplant phase she was admitted in her local hospital with recurrent fevers and developed new cardiac murmurs and was subsequently diagnosed with *A. fumigatus* endocarditis 8.6 months post allograft. She also had pulmonary CT signs (nodules) and space occupying lesion in the brain suggestive of IA. It is worth noting that she had fully sensitive *A. fumigatus* and despite combination

antifungals she succumbed to her disease. The total antifungal cost was £117,279.20 (£3,421.96 on prophylaxis and £113,857.20 on treatment).

Prolonged follow-up is necessary to capture this type of long term events. Most of this took place in her local hospital and was admitted in ITU in a neighbouring trust. Alemtuzumab is associated with profound and prolonged immune suppression and may explain the longer at risk period in this patient.[30]

Case 8: This was the only case of proven fusariosis in our cohort and indeed the only case identified among the King's haematology patients in the last 20 years. She had two primary graft failures from different donors and unsurprisingly was persistently pancytopenia for a prolonged period of time. Fusariosis is associated with poor prognosis.[372,373]

Chapter 7 Summary & Conclusions

7 Summary & Conclusions

- The incidence of IFD by using a comprehensive diagnostic approach is higher than reported in most studies in the literature. This approach bears closer resemblance to post mortem data. In order to demonstrate the true incidence of IFD two things are required. The first is the use of all available tools that are recognized for the diagnosis of IFD. Relying on a single mycological test such as GM can lead to misleadingly low rates. Secondly, prolonged follow-up is necessary to truly capture the at-risk period.

- The cumulative incidence of IFD at 3, 6, 12, and 24 months was 16, 19, 21, and 21% respectively (Figure 35). The cumulative incidence of IA at 3, 6, 12, and 24 months was 14, 16, 17 and 18% respectively. The proven cases were *A. fumigatus* (2), moulds NOS (5), *Fusarium spp.* (1), *Pneumocystis jiroveci* (1), non-*Candida albicans spp.* (5). The median time from index treatment to onset of IFD was 142, 17, and 41 days for proven mould disease, candidiasis and probable IFD, respectively.

- The index treatment, identified as the treatment during which IFD occurred, was chemotherapy (21: induction 16, consolidation 4, aplasia post consolidation 1), allogeneic HSCT (17), IST (4: pre-IST aplasia 2, IST 2) and autologous HSCT (2). Allograft/chemotherapy/IST were associated with the highest risk of IFD with treatment-specific incidences of 16, 14, and 12%, respectively. Autograft was associated with low risk of IFD (3%).

- Bacteraemia was seen in 74 (36%) of this cohort. These organisms were Gram positive cocci (27; 36%), Gram negative rods (25; 34%), mixed (20; 27%) and non-tuberculous mycobacterial spp (2; 3%). Concomitant bacteraemia was seen in 18 (9%) of the study patients.

- Antifungal therapy was given to 101 (50%) patients during the course of the study emperically. The median duration of treatment was 32 days (interquartile range 8-80 days; range 1-456 days). The percentage of patients who were treated for 2 weeks or more (33%) was similar to the combined incidence of proven/probable/possible IFD (70/209; 33%) and also to autopsy data[77].

- The sensitivity, specificity, PPV, and NPV for IFD detection by GM assay was 54, 71, 82, and 39% respectively. The coresponding values for BDG assay were 79, 55, 83, and 49%; whole blood PCR were 15, 97, 75, and 73%; serum PCR were 35, 96, 94, and 40%; LFD 9, 98, 44, and 88%, respectively. The sensitivity of biopsy was 35%. The proportion of caes truly identified by both GM and BDG was 38%, GM and LFD 9%, BDG and LFD 22%. Serum and blood PCR were dually positive in 24% of cases of proven/proable IFD. No single test was able to detect all the cases but combining the results from BDG and GM assays seemed to offer the best biomarker combination.

- Biopsy is feasible and safe during intensive chemotherapy or HSCT. Tissue diagnosis remains the gold standard for the correct diagnosis or

exclusion of IFD. Given the safety of the procedure, clinicians should perform this more often in patients who are fit for biopsy once their pre-operative parameters are optimised. However, better localization techniques are required to obtain adequate samples and therefore avoid non-specific results.

- Baseline CT abnormalities were detected in 38% of patients which preceded neutropenia and signs and symptoms of sepsis and ahead of positive mycological results. These abnormalities, especially EORTC-signs (HR 4.3; 95% CI 1.9-9.8; P<0.001), were associated with significantly increased risk of IPA in a multivariate model. This should form part of the initial screening before the initiation of chemotherapy or HSCT.

- Other independent risk factors were baseline MCP-1 >841 pg/ml (HR 2.7; 95% CI 1.2-6.1; P=0.016), baseline IL-2R >834 pg/ml (HR 2.3; 95% CI 1.1-5.1; P=0.037), baseline Karnofsky score <90 (HR 2.1; 95% CI 1.1-4.2; P=0.034), monocytopenia (<0.1 $x10^9$/L) for >10 days (HR 2.6; 95% CI 1.3-5.4; P=0.009), and bacteraemia during the treatment period (HR 2.5; 95% CI 1.2-5.0; P=0.013).

- No statistically significant differences in follow-up cytokines were found between proven/probable IFD and those with no evidence of IFD. Baseline and follow-up cytokines had no prognostic significance.

- The overall survival (OS) estimate at 3, 6, 12, and 24 months were 91%, 81%, 72% and 68%, respectively for the whole cohort and 92%, 83%, 76%,

and 70%, respectively for the IA cohort. The 2-year survival estimates (95% CI) were 45 (29-61)%, 66 (56-76)%, and 87 (77-97)%, for proven/probable, possible/not classified and no evidence of IFD respectively. The corresponding estimates for the IA cohort (N=195) were 52 (34-70)%, 66 (56-76)%, and 87 (79-95)% for proven/probable IA, possible IA/not classified, and no evidence respectively.

- Relapse or progression of underlying haematological malignancy was the commonest cause of death (59%) while IFD was the second commonest cause accounting for 20% of deaths. The IFD-attributable mortality was 59% for patients who died in remission without GVHD but the overall IFD-attributable mortality was 82%. Therefore, proven/probable IFD carries considerable mortality.

- The factors associated with prognosis were ITU admission, allograft/chemotherapy/IST, AML/MDS, bacteraemia, hepatic dysfunction, renal impairment, and proven/probable IFD.

- Case 2 high-lights the difficulty of making IFD diagnosis. As CT scan interpretation is observer-dependent it is important to have chest radiologist review even though this may be challenging in busy clinical settings and where a trained chest radiologist may not be available. The importance of autopsy examination was clearly demonstrated here, unifying disparate clinical features into a single diagnosis, albeit remarkably disseminated.

Chapter 8 Future Directions

8 Future directions

Having demonstrated the incidence and risk factors of IFD in this cohort which represents 85% of eligible cases, a number of key diagnostic approaches need further development.

1. Serodiagnostics

   It is clear from the results of this study that a single assay is insufficient and the best combination is GM and BDG. We now need to establish how best to use these in combination. In the study GM was done twice a week on all patients while BDG was done on a selection of cases. The cost of BDG is an important consideration (current market price/test is GM £8.43, BDG £47.38). There are three different options:

   i. GM twice weekly on all during the at-risk period and BDG on positive GM and CT abnormalities both with and without EORTC signs.

   ii. Surveillance testing using both GM and BDG assays

   iii. BDG twice weekly and GM on positive sera and CT signs.

   Option one is most logical and cost-effective.

2. Radiology

The finding of 38% prevalence of pre-therapy chest CT abnormalities is important. Although it is an independent risk factor for IPA, its role in the incidence of IPA is not established. A clinical trial randomising patients with baseline abnormalities to upfront 'pre-emptive' antifungal therapy vs. continuing antifungal prophylaxis until clinical indication for therapy (standard of care) would be an important management strategy.

The main drawback for CT in the diagnosis of IFD is its lack of specificity. A novel *in vivo* imaging method involving the use of ^{68}Ga-labelled siderophores internalized by the fungus and the site of infection, which are subsequently detected by positron emission tomography scanning, is an important advance.[374,375] The early *in vitro* and animal data are promising but need to be translated into the clinic.

3. Bronchoscopy

There is increasing evidence for the diagnostic value of bronchoscopy and BAL GM and BDG.[303,376-381] The GMI cut-off remains a matter of debate[303,382] Although a positive BAL culture does not distinguish between colonisation and infection, it is highly suggestive of the latter in patients at high risk of IFD.[383] In addition it may also provide evidence of bacterial or viral infection.[333]

The low bronchoscopy rate in this study reflects the clinical reality within the wider haematology patients. Renewed efforts need to be made to enable timely bronchoscopy service to these patients by linking more closely with the respiratory physicians and identifying interested individuals.

4. Biopsy

Although minimally invasive approaches such as VATS provide an opportunity for obtaining tissue diagnosis the high rate of inconclusive biopsy results is a worry. Moreover, VATS is not suitable for centrally located lesions or large lesions greater than 3 cm.[384] The methylene blue localisation technique used here was also disappointing as it did not improve the diagnostic yield although the numbers were small. The right surgical technique is at the discretion of the cardiothoracic surgeons but other localisation techniques such as image-guided placement of a hook-wire[281,282] or platinum microcoils[283] could be further explored with the surgeons.

5. TDM & Changes in antifungal protocol

Now that local capacity has been developed TDM should be incorporated into local protocols to ensure that adequate recommended therapeutic

levels are achieved. Moreover, the incidence of IFD should be reinvestigated again to assess the impact of changes in local protocol which recommends posaconazole for all high risk patients.

6. Genetic risk profiling

Genetic susceptibility to IFD based on mutations of key innate immunity genes such as Dectin-1[66,121], TLRs[62] and cytokines[67,68] hold promise in providing some tools for better susceptibility profiling. This should form part of subsequent studies.

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Appendix 1 Publications from this Thesis

Paper

Ceesay MM, Desai SR, Berry L, Cleverley J, Kibbler CC, Pomplun S, Nicholson AG, Douiri A, Wade J, Smith M, Mufti GJ, Pagliuca A. Galactomannan Surveillance Combined With Targeted β-D- Glucan Improves The Diagnostic Accuracy of Invasive Fungal Disease in Adult Neutropenic Hemato-oncology Patients. *In press*

Abstracts

Ceesay MM, Berry, Desai SR, Cleverley, Kibbler CC, Wade J, Smith M, Muft GJ, Pagliuca A. A Comprehensive Diagnostic Approach Improves the Diagnostic Accuracy of Invasive Fungal Disease (IFD) in Adult Haemato-Oncology Patients Undergoing HSCT or High Dose Chemotherapy- Results of the King's Prospective Aspergillosis Study (NCT00816088). Oral session; 2012 BSH 52nd Annual Scientific Meeting.

Buckner SL, **Ceesay MM,** Berry L, H Sikondari H, Smith M, Wade J, Pagliuca A, Flanagan RJ. Therapeutic drug monitoring of itraconazole and Posaconazole. Oral Session Two; 2011/03/08 16:45:05.

Ceesay MM, Berry L, Desai SR, Marrinan M,Deshpande R, Whitaker D, Pomplun S, Nicholson A, Sikondari H, Wade J, Smith M, Mufti GJ, Pagliuca A. Video-assisted thoracoscopic (VATS) biopsy is feasible and safe in adult haemato-oncology patients undergoing stem cell transplantation or high dose chemotherapy BSH 2011 oral session Two. 2011/03/08 16:45:05.

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Appendix 2 Database variables (summary)

1. Baseline information

   Patient ID age sex ethnic origin weight smoking history alcohol history previous therapy date of admission reason for admission Karnofsky score PAM score (allografts) disease status (CR/PR/Stable/progression/new diagnosis) lung function (FEV1, TLCO) baseline CT results GvHD (yes/no) co-morbidities FBC LFT U&E CRP

2. Clinical assessment (follow-up)

   Treatment (chemotherapy/allograft/IST) fever pleuritic chest pain GvHD (acute/chronic and grade)

3. Antibiotics

   Drug dose reason for starting reason for stopping

4. Antifungals

   a) Prophylaxis (drug, dose, reason for starting [primary or secondary unit protocol, other], date of starting and stopping)

   b) Treatment (drug, dose, reason for starting [empiric, possible, probable, proven IFD] and stopping)

5. Antivirals

   Drug dose reason for starting reason for stopping

6. Other immunosuppressive agents including steroids, dose, duration, reason

7. Galactomannan (date, GMI, positive/negative)

8. B-D glucan (date, results)

9. PCR (date, type, results)

10. CT (date, results of baseline/diagnostic/follow-up 1 or 2)

11. FBC (Hb, WBC, Neutrophils, platelets, monocytes, lymphocytes; duration of neutropenia, monocytopenia, and lymphopenia)

12. LFT (bilirubin, AST, ALP, GGT, albumin)

13. U&E (urea, creatinine, electrolyes)

14. CRP
15. EORTC status (& date)
16. Outcome (alive/dead, cause of death, overall survival)
17. Blood culture results
18. Viral PCR results
19. Bronchoscopy results
20. Biopsy results